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A METHOD FOR THE QUANTITATIVE SPECTROCHEMICAL ANALYSIS OF VERY SMALL AMOUNTS OF BIOLOGICAL MATERIALS FOR SODIUM, POTASSIUM, CALCIUM, MAGNESIUM, AND LEAD

BY O. S. DUFFENDACK, K. B. THOMSON, WILLIAM C. LEE, AND
O. G. KOPPIUS

(From the Department of Physics, University of Michigan, Ann Arbor)

(Received for publication, July 12, 1938)

In a previous paper (1), two of the authors described a spectrographic method for the analysis of biological fluids, urine in particular, for sodium, potassium, calcium, and magnesium. This method gives highly satisfactory results, but it was found in practice that the amount of material required was sometimes inconveniently large, especially for some fluids. The present paper describes a method developed for analyses when the amount of material available is too small for the former method. Additional advantages arising from the ease of operation will be apparent and in our practice the method here described has completely displaced the former one.

The essential difference in the two methods lies in the source used for the excitation of the spectrum. In the present method, the source used is a special type of electric arc which has been briefly described in an earlier paper (2) and more completely in a recent one (3). Carbons of the highest purity 0.5 inch long and 0.25 inch in diameter are used. Each carbon has one end filed off to a smooth flat surface, and a single drop of the liquid to be analyzed is dried on the smooth end of each of two carbons. The drying should be sufficiently rapid to prevent the liquid from soaking into the carbons. The carbons are used as electrodes for the arc which plays between the dried salts on the ends of the electrodes.

The arc is a high voltage, low current a.c. arc with some peculiar properties. A potential difference of 1100 or 2200 volts and a current of about 2.2 amperes have been found satisfactory. The

power is supplied by a 5 kilovolt-ampere step up transformer such as is used in city service distribution. The impedance for controlling the current is put in the secondary circuit in series with the arc. This impedance may be pure ohmic resistance or may be chiefly inductive reactance. In the latter case, we have employed choke coils with a resistance of 100 ohms and a reactance of about 450 ohms at 60 cycles. Marked changes can be produced in the behavior of the arc and in the relative intensities of the spectral lines by varying the inductance, and the most favorable combination of resistance and inductance is used. With the choke coils described, a steady arc can be maintained at 1100 volts across a gap of 2.5 mm. as long as there is salt on the electrodes. The arc will not maintain itself on clean carbons or on carbons which are coated with salts that do not contain a sufficient amount of the alkali metals. The arc does not develop nearly as much heat at the electrodes as does an ordinary d.c. arc. After several minutes operation, the electrodes show no signs of redness, and as a result of this low temperature the salt remains on the carbons throughout the exposure.

The arc current and the length of the gap require very close regulation. The length of the gap was found to be a particularly important factor, and a special arc stand has been designed to give a constant arc gap without an undue amount of care in adjusting it. This apparatus has been described in a recent paper (3) to which reference may be made for details. The arc current should be maintained constant to within ± 0.05 ampere.

Analysis for Sodium, Potassium, Calcium, and Magnesium

The material to be analyzed was prepared by ashing it in silica vessels with sulfuric acid. In a previous paper (1), it was pointed out that this procedure led to an apparent loss of potassium but the amount is small and negligible except for the highest precision. A complete ashing was found to be necessary because the presence of a small amount of organic material caused the liquid to soak into the carbons much more rapidly. The ash was dissolved in a solution which contained 2 gm. of lithium and 0.05 gm. of chromium per 100 cc., added as chlorides. In addition to these elements, the solvent contained sulfuric acid (0.05 N), hydrochloric acid (0.05 N), and phosphoric acid (0.03 N). The latter was added because previous experiments showed that the

presence of a small amount of phosphate had a rather marked effect on the relative intensities of certain lines, but that this effect became constant for values of phosphate concentration of the order of magnitude given by the above quantity of phosphoric acid. It was much easier to add this quantity than to remove the phosphates from the samples. The lithium and chromium were used as internal standards, and the lithium also supplied the alkali salt necessary to make the arc burn properly.

Analyses were made for sodium, potassium, calcium, and magnesium by this method with an arc of 2.2 amperes at 1100 volts across a 2.5 mm. gap. Some analyses were made for copper also, but the results were not very conclusive because of the presence of copper in the chemicals used and in the walls of the glass vessels used for storing the solutions. The source is very sensitive for copper, and, for the determination of minute amounts of copper, it will be necessary to use copper-free reagents and vessels as was done in the case of lead described in the next section.

The analytical procedure is like that described in the previous paper (1). The magnesium spark line at 2795 Å. was compared with the chromium spark line at 2835 Å.; potassium 4044 Å. was compared with lithium 3985 Å.; and sodium 3303 Å. and copper 3247 Å. with lithium 2741 Å. The logarithm of the ratio of intensities of the selected pair of lines was found by microphotometer measurements on a plate calibrated by means of a stepped slit after the manner described by Thomson and Duffendack (4). These logarithms were plotted against the logarithms of the concentrations for a series of prepared solutions of known concentrations to give the analytical curve. After the relative intensities of the lines of the test elements and internal controls are determined for an unknown solution, the values are applied to the analytical curve from which the concentrations of the unknown solution are read.

Investigation showed that the effect of one element on the analysis for other elements was very much smaller with this source than with the jet source (1), so that these effects could be neglected except for the effect of phosphorus. The effect of phosphorus was eliminated, as pointed out above, by adding sufficient phosphoric acid so that any changes in the concentration of phosphate produced no changes in the relative intensities of the lines.

In testing the method, a number of solutions were made up and

analyzed. The percentage difference between the values found by analysis and the actual values for seven such solutions, each of which was analyzed five times, were as follows: for potassium, twenty-eight of the thirty-five analyses were within 5 per cent of the correct values, and three were more than 10 per cent off; the average error was 4 per cent, the largest 17 per cent; for magnesium, eighteen of the thirty-five were within 5 per cent and six were off by more than 10 per cent with an average error of 6 per cent. The results for sodium were somewhat more erratic and had an average error of 8 per cent. Since this work was done, the technique has been improved and more accurate results can now be obtained (3), and calcium, which could not be satisfactorily determined with the 1100 volt arc across a 2.5 mm. gap, can also be measured accurately with a 2200 volt arc across a 0.5 mm. gap, as described in the paper referred to above.

Most of the practical work with this method has been done on saliva. 5 cc. of saliva were ashed and dissolved in 1 cc. of the solution described above. A wide range of concentration was found for all of the elements for which analysis was made. The following values are roughly the limits of concentration found.

	<i>gm. per l.</i>	
Magnesium.....	0.001	-0.01
Sodium.....	0.07	-1.5
Potassium.....	0.15	-1.0
Calcium.....	0.02	-0.1
Copper.....	0.0005	-0.002

The potassium values showed a remarkable correlation with the condition of the teeth (5), and further research is now being carried on by the Dental School of the University on the relation of the amount of potassium in saliva to dental caries. The method is applicable to practically all body fluids, and it has been used for the determination of lead in skin, hair, finger nails, and urine of humans, and liver, kidney, spleen, muscle, and other tissues of guinea pigs. The amount of material required is limited practically by the difficulty of measuring out very small volumes. Samples of fluids as small as 0.1 cc. have been analyzed successfully.

Analysis of Urine for Lead

A method for analyzing urine and tissue for minute traces of lead was developed at the request of two departments of the

Medical School of the University. The procedure is similar to that described above except that it has been found more convenient and advantageous to oxidize the organic materials in urine by means of hot nitric acid in preparation for the analysis for lead.

The preparation of the sample is as follows: To a 100 cc. Kjeldahl flask are added 5 cc. of urine and 3 cc. of a solution containing 0.01 gm. of sodium as sodium nitrate and 0.06 mg. of bismuth as bismuth subnitrate per cc. The sodium is for the purpose of supplying alkali to the arc to make it burn properly and the bismuth is the internal control. This solution is evaporated to dryness and gives a black residue because of the decomposition

TABLE I
Determination of Lead in Urine Specimen

Sample No.	$\text{Log } \frac{I_{\text{Pb}}}{I_{\text{Bi}}}$	Lead
		<i>mg. per l.</i>
1	-4.20	0.34
	-4.15	0.35
2	-4.17	0.34
	-4.13	0.35
3	-4.18	0.34
	-4.19	0.34
4	-4.16	0.34
	-4.30	0.32
5	-4.10	0.35
	-4.20	0.34

of the organic materials. Concentrated nitric acid is now added a drop at a time to the hot flask until the material turns white, and then the excess nitric acid is boiled off. The residue is then redissolved in 0.5 cc. of distilled water. A drop of this solution is placed upon the smooth ends of each of two carbons as described before and these then are used as the electrodes for the arc. A 2.5 ampere arc at 2200 volts, 60 cycle A.C. is maintained across a gap of 0.5 mm. and the spectrum is photographed with a medium size quartz spectrograph. A condensing lens is used between the arc and slit to reduce the time of exposure. For the analysis the lead line at 2833 Å. is compared with the bismuth line at 2898 Å.

A typical set of data is given in Table I for one urine specimen

to show the reliability of the method. Two duplicate analyses of each of five different samples of the same specimen were made. The analytical curves that have been determined enable us to measure lead in concentrations down to 0.1 mg. per liter of urine. Below this value the analyses lose considerably in accuracy.

The importance of the preparation and maintenance of lead-free reagents for this work cannot be overemphasized. The water was triply distilled in a Pyrex still and stored in glass bottles lined with paraffin. The concentrated nitric acid was likewise triply distilled in a Pyrex still and stored in Pyrex flasks. After long standing, it was found necessary to redistill the nitric acid. The sodium was added as sodium nitrate that had been prepared from lead-free sodium hydroxide and lead-free nitric acid. The bismuth subnitrate was tested and found to be lead-free. All of the reagents were tested before use by examining their spectra when excited by the source used for the analyses. The internal standard solution containing sodium nitrate, bismuth subnitrate, lead-free water, and a slight excess of nitric acid was stored in a paraffin-lined bottle and was found to remain pure for a long time. All of the glassware was rinsed with distilled water, lead-free nitric acid, and lead-free distilled water before being used for the analysis.

The method described above has certain advantages in case of manipulation. After an analytical curve has been determined, only two quantities need to be measured accurately in making an analysis; namely, the 5 cc. of urine and the 3 cc. of the internal standard solution. The amount of nitric acid taken to oxidize the organic material and the amount of lead-free water used to redissolve the residue need not be kept constant, because the ratio of the amount of lead to the amount of bismuth present remains the same at any dilution. Two duplicate analyses of each of four specimens can be made in approximately 2 hours.

SUMMARY

A method of spectrographic analysis for sodium, potassium, calcium, magnesium, and lead in biological fluids has been described, which has proved useful in practice. One of the chief advantages of the method is that a very small amount of material is required; only 2 drops are necessary, although it is usual to

prepare larger amounts for convenience in the volumetric measurements. Another advantage is its wide adaptability. It may be used for almost any biological fluid or tissue without alteration, but tests should be made for the effects of radical changes in the concentration of any elements on the intensities of lines of other elements. This spectroscopic source has proved useful in a variety of other practical problems.

The authors wish to acknowledge their indebtedness to the Rockefeller Foundation for financial aid in the first part of this investigation.

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QUANTITATIVE STUDIES OF CARNOSINE AND ANSERINE IN MAMMALIAN MUSCLE*

I. A METHOD FOR THE DETERMINATION OF CARNOSINE AND ANSERINE

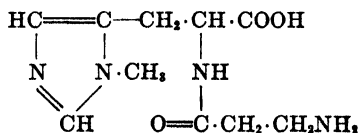
BY JOHN A. ZAPP, JR., AND D. WRIGHT WILSON

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, July 2, 1938)

Carnosine was first isolated from beef muscle extract by Gulewitsch and Amiradzibi (10) in 1900, and has subsequently been shown to have a wide-spread occurrence in mammalian skeletal muscle. The investigations of Gulewitsch (8, 9), Baumann and Ingvaldsen (3), and Barger and Tutin (2) proved that carnosine is β -alanyl-L-histidine.

Anserine was isolated from an extract of goose flesh by Ackermann, Timpe, and Poller (1) in 1929. Linneweh, Keil, and Hoppe-Seyler (22), Keil (16), and Linneweh and Linneweh (23) were able to show by various degradations that anserine is a monomethylcarnosine whose formula is



This compound has been synthesized from L-1-methylhistidine by Behrens and du Vigneaud (4) and its identity with natural anserine established.

* This paper together with the paper following has been presented by John A. Zapp, Jr., to the Faculty of the Graduate School of the University of Pennsylvania as a dissertation in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

A preliminary report of this work was presented before the American Society of Biological Chemists, at Baltimore, April 2, 1938 (35).

10 Carnosine and Anserine in Muscle. I

It was first thought that anserine occurred only in the muscles of birds and certain reptiles, and that it was absent from mammalian muscle (16). However, anserine has been isolated from the muscles of various mammals by Wolff and Wilson (32, 33) and later by other investigators (21, 25, 6). In most cases, both carnosine and anserine were found to be present in muscles of the same species.

The relatively high concentration of carnosine or anserine, or both, in all vertebrate skeletal muscles examined for these compounds, suggests that they are physiologically important constituents. However, their function is unknown. Hunt and du Vigneaud (11) have recently reported that the slight depressor effect of carnosine is due to the β -alanine part of the molecule. Du Vigneaud, Sifferd, and Irving (29) demonstrated that carnosine will support growth in rats on a histidine-deficient diet. It seems unlikely that either the depressor or growth-supporting actions can account for the normal metabolic function of the compounds.

The isolation of carnosine and anserine is tedious, requires large amounts of material, and is attended by considerable losses. Gravimetric, colorimetric, and titrimetric procedures for the quantitative determination of carnosine in muscle extract have been proposed. Most of these methods were reviewed by Wilson (30) in 1932. The Koessler and Hanke (19) colorimetric diazo procedure for imidazoles has been the choice of most investigators. Hunter's (12) critical studies indicate that carnosine is responsible for at least 95 per cent of the diazo color in a protein-free extract of fresh mammalian skeletal muscle.

Anserine gives no color with the diazo reagent. However, a method for the estimation of carnosine and anserine in muscle, through the colorimetric determination of histidine and 1-methylhistidine in hydrolyzed muscle extract, has been proposed by Kapeller-Adler and Haas (15). The method is based on the Knoop reaction of certain imidazoles with bromine. Since 1-methylhistidine gives a color similar to but one-fifth as intense as histidine with this reaction, and since histidine but not methylhistidine reacts with the diazo reagent, the two compounds may be determined by using the two colorimetric procedures.

In testing this method on pure histidine solutions, our best

results varied by about 10 per cent. In the original procedure for histidine, Kapeller-Adler (14) stresses the necessity for exact bromination to a pale permanent yellow, since any marked excess of bromine causes low results, presumably because of oxidation. However, this oxidation does not take place if the excess bromine is removed before the solution is made alkaline.¹ We found it more convenient, therefore, to overbrominate slightly and to remove the surplus bromine with phenol before addition of the ammonia mixture. Conrad and Berg (5) and Woolley and Peterson (34) have reported similar procedures in which the excess bromine is removed with arsenious oxide, and by aeration, respectively. There are other factors which may cause change in shade and intensity of color with pure histidine solutions. Perhaps the most important of these are: (1) the amount of ammonia mixture added per volume of a given histidine solution and (2) the length of time during which the alkaline solution stands at room temperature before heating. While these variables merely emphasize the necessity for a well standardized procedure, other difficulties are encountered when dealing with muscle extracts. β -Alanine must be thoroughly removed, since it interferes with the determination to the extent that hydrolyzed muscle extract or hydrolyzed carnosine fails to give a Knoop reaction until a mercury precipitation (as suggested by Kapeller-Adler and Haas (15)) or similar separation of histidine (and methylhistidine) is performed. Woolley and Peterson (34) list a number of amino acids which interfere with the Knoop reaction. The Kapeller-Adler and Haas procedure also calls for the use of KMnO_4 to decolorize the muscle extract, and this invariably leads to rapid fading of color. In our hands, the method led to such erratic results for anserine that we felt it to be untrustworthy.

Wilson and Wolff (unpublished work) studied copper colorimetric and copper gravimetric methods for anserine, and found them to be unsuitable. In 1933, Wilson and Wolff (31) published a preliminary report on a method for the estimation of anserine, which was based on a determination of the increase of α -amino nitrogen resulting from the hydrolysis of carnosine and anserine. Carnosine was determined by the diazo procedure. This method ap-

¹ For a suggested mechanism of the oxidation reaction, see Woolley and Peterson (34).

peared to be the most promising of those available, and with some modifications and extensions it is reported in the present study.

Our procedure may be outlined as follows: Carnosine and anserine are precipitated from a protein-free muscle extract by mercuric acetate and alcohol. The mercury salts are decomposed with H_2S , the mercuric sulfide is removed, and the centrifugate and washings are concentrated to a suitable volume. Carnosine is then determined on an aliquot of this solution by the diazo method. Other aliquots are used for the determination of α -amino nitrogen both before and after hydrolysis. One amino group of carnosine and one of anserine react in the Van Slyke amino nitrogen determination in the time required for α -amino groups. After hydrolysis, another amino group reacts. In the absence of other peptides, the increase in α -amino nitrogen on hydrolysis may be assumed to be due to amino nitrogen set free by the hydrolysis of carnosine and anserine.

The accuracy of the anserine determination depends, among other things, on (1) a correct value for carnosine and (2) the absence of all peptides other than carnosine and anserine from the final extract. In the latter connection, glutathione appears to be the peptide most likely to interfere. Indeed, Krause (20) reported that in the bovine lens, retina, and vitreous humor there was sufficient glutathione, as calculated from the cystine content of the hydrolysates, to account for all the increase in amino nitrogen other than that due to carnosine. In our studies, we ran cyanide-nitroprusside tests routinely, and found only traces of sulfhydryl in the final extracts. In a few cases we tried the more specific nickel-nitroprusside test, recommended by Zimmet (36) for the detection of glutathione, with similar results. In one sample a Sullivan test on the hydrolysate indicated that no cystine was present. In dealing with tissues which contain an appreciable amount of glutathione, it may be feasible to remove the glutathione as the cuprous mercaptide (Pirie and Bernal (26)).

A routine biuret test was made on the final extracts to confirm the complete removal of the higher polypeptides. Theoretically (17, 18), one should expect a biuret color from all peptides containing three or more amino acids. In the face of a negative biuret test, and of a negative cyanide-nitroprusside test, it seems reasonable to assume that the only peptides present are carnosine

and anserine. We are aware of no evidence that other dipeptides or piperazines, precipitable by mercury, occur in fresh mammalian muscle in amounts large enough to interfere with our method.

Method

Samples of muscle are dissected within a few hours after death, and are either extracted immediately or refrigerated for not more than 24 hours before extraction. The muscle is freed from excess fat and connective tissue, and run through a grinder until well minced. A weighed portion (usually 15 to 50 gm.) is then extracted three times at pH 5 to 6 with 3, 3, and 2 parts of acidified water for $\frac{1}{2}$ hour each at 60–70°. By this procedure, all but a trace of the imidazoles are extracted, while most of the protein is precipitated. The combined extracts are concentrated on a water bath, below the boiling point and before a fan, to an accurately known volume representing about 1 gm. of muscle per cc. This procedure is as satisfactory as vacuum distillation for extracts of mammalian skeletal muscle, and is usually more convenient. A small coagulum forms during the concentration, and is removed and washed by centrifugation. An aliquot of the resulting solution is then taken, the pH is adjusted to between 5 and 6, and 5 parts by volume of boiling 95 per cent alcohol are added. The solution is then heated to boiling on a water bath. Final traces of protein and some glycogen are precipitated. Heating the alcoholic solution causes coagulation of the alcohol-insoluble proteins, makes filtration easy, and reduces the chance of adsorption of nitrogenous extractives on the coagulum. The concentration of alcohol should not be increased beyond the amount stated, as there would then be danger of precipitating some of the carnosine and anserine. The alcoholic filtrate and washings are neutralized to litmus with NaOH, and excess solid mercuric acetate is added to effect a separation of carnosine and anserine from some of the other extractives. The mercury precipitate is centrifuged, washed, suspended in hot 0.1 N H_2SO_4 , and decomposed with H_2S . The mercuric sulfide is removed by centrifugation and is washed repeatedly with 0.1 N H_2SO_4 . Sulfate is then removed from the combined centrifugates with barium hydroxide, the barium sulfate is removed and washed, and the

centrifugates are concentrated to an accurately known volume representing about 1 gm. of muscle per cc.

Carnosine is then determined on an aliquot of this solution by the diazo method. The procedure used is essentially that of Koessler and Hanke (19) with the additional modifications that a blue-green filter (Wratten No. 75) is used in the colorimeter as suggested by Eggleton and Eggleton (7), and the test solutions are read against Hunter's (13) dye standard. The color value of a sample of carnosine nitrate was found to be identical with the average color value for carnosine reported by Hunter (13) (141×10^6 mm. per mole, in Hunter's units).

Other aliquots are used for the determination of α -amino nitrogen by the Van Slyke manometric method, both before and after hydrolysis. For complete hydrolysis of carnosine and anserine, the solution is heated for 2 hours at 100° with 0.5 volume of concentrated HCl. Muscle extract contains compounds which react slowly with nitrous acid. These may be corrected for by the procedure recommended by Van Slyke and Meyer (28), in which the reaction is carried out for twice the time necessary to decompose the α -amino groups. This correction must be made for both unhydrolyzed and hydrolyzed muscle extracts.

The increase in α -amino nitrogen on hydrolysis is assumed to be due to amino nitrogen set free by the hydrolysis of carnosine and anserine. Although carnosine and anserine give slightly more amino nitrogen per mole than would be expected by theory, the *increase* in amino nitrogen on hydrolysis should be of the expected theoretical magnitude, since it comes from the newly liberated histidine which gives the calculated amount of amino nitrogen in the Van Slyke determination. Therefore, the increase in amino nitrogen after hydrolysis should correspond to 14 gm. per mole of either carnosine or anserine. That this is demonstrated to be true within the limits of experimental error may be seen from Table I. Consequently, anserine may be found by the following calculation. Anserine = $(B - A) \times (240/14)$, where B = total increase in α -amino nitrogen on hydrolysis and A = increase in α -amino nitrogen due to hydrolysis of carnosine (calculated from the diazo value).

A complete determination takes about 5 days. It is well not to allow a muscle to stand more than 24 hours after death, even

on ice, before extracting. Carnosine and anserine may disappear slowly from refrigerated muscle and there is always a possibility of slight proteolysis which might lead to the formation of polypeptides not precipitable with alcohol. The mercury precipitates, however, have been kept in the ice box for some weeks without appreciable loss of carnosine or anserine.

The results obtained for carnosine in protein-free muscle extract are probably fairly accurate, since guanine, xanthine, histidine, and tyrosine are the only known non-protein muscle constituents likely to interfere with the diazo determination. Of these compounds, guanine and xanthine are probably present

TABLE I
Determination of Amino Nitrogen in Carnosine and Anserine

Preparation	Before hydrolysis		After hydrolysis		Increase after hydrolysis		
	Calculated	Found	Calculated	Found	Calculated	Found	$\frac{\text{Found}}{\text{Calculated}} \times 100$
	per cent	per cent	per cent	per cent	per cent	per cent	
Copper carnosine.....	4.58	4.72	9.16	9.34	4.58	4.62	101
Carnosine (base).....	6.19	6.44	12.38	12.50	6.19	6.06	98
Copper anserine.....	4.38	4.52	8.76	8.94	4.38	4.42	101
Anserine nitrate.....	4.62	4.71	9.24	*	4.62	*	

* Deamination occurred during hydrolysis.

only in traces in extracts of mammalian muscle (Ostern (24)), and histidine has not been demonstrated in fresh mammalian muscle. Sifferd and du Vigneaud (27) have isolated tyrosine from sheep muscle, and suggest that this may be the "undetermined substance," which, according to Hunter (12), is responsible for about 2 per cent of the diazo color in mammalian muscle extract.

Samples of carnosine and anserine were added separately to muscle extracts in order to study their recovery by the method. The results are shown in Table II. The carnosine recoveries, calculated from the diazo value after mercury precipitation, are

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from 91 to 97 per cent of the carnosine added. The recoveries of added anserine are 102 and 107 per cent. In each of the four experiments in which carnosine was added to a muscle extract, anserine was determined on the original extract and on the portion containing the extra carnosine. These anserine determinations are therefore duplicates, and give some indication of the variations which may occur in two estimations on the same muscle.

TABLE II
Recovery of Added Carnosine or Anserine

	Ox No.	Muscle	Carnosine (diao)	NH ₄ -N increase after hydrolysis	Anserine (B - A)	Carnosine or anserine added	Carnosine found	Anserine found	Per cent recovery
			A	B					
			Mg. NH ₄ -N per 100 gm. muscle			Mg. substance per 100 gm. muscle			
Carnosine	1	Glossus*	16.9	23.9	7.0		273	120	91.4
		"	22.8	29.2	6.4	105	369	110	
	2	"	17.1	18.7	1.6		276	27	
		"	23.2	24.5	1.3	102	375	22	
	3	Buccinator	0.9	4.1	3.2		15	55	
Anserine		"	17.7	22.8	5.1	278	285	87	97.1
	4	"	1.2	4.3	3.1		19	53	94.7
		"	17.5	21.2	3.7	278	283	63	
	5	Glossus	16.9	24.6	7.7		273	132	107.0
		"	17.2	33.3	16.1	134	278	276	
	6	"	17.9	23.5	5.6		289	96	
		"	16.9	28.4	11.5	99	273	197	102.0

* "Glossus muscle" is used to refer to muscle taken from the genio-glossus, hyoglossus, and styloglossus muscles, without differentiation.

In the experiments in which anserine was added, the carnosine determinations on the original extract and on the aliquot with added anserine constitute duplicate determinations on the same muscle.

SUMMARY

A method is proposed for the determination of carnosine and anserine in mammalian muscle. Carnosine is determined by the

dialyso method after mercury precipitation, and anserine is calculated from the increase in α -amino nitrogen after hydrolysis.

The determinations are based on reactions which are not entirely specific. Presumably the determination of anserine is more open to error than that of carnosine, since the anserine values will be increased if other peptides are present in the mercury precipitate of the muscle extract. While the figures yielded must be considered more or less tentative, reasons are presented in this and the following paper for believing that they are of value.

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QUANTITATIVE STUDIES OF CARNOSINE AND ANSERINE IN MAMMALIAN MUSCLE*

II. THE DISTRIBUTION OF CARNOSINE AND ANSERINE IN VARIOUS MUSCLES OF DIFFERENT SPECIES

BY JOHN A. ZAPP, JR., AND D. WRIGHT WILSON

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

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For comparative purposes, a knowledge of the distribution of carnosine and anserine in various muscles of an animal, and in the muscles of different members of the species, is of interest. The results of several investigators may be compared with respect to the amount of carnosine found by analysis in various muscles of the cat, but the published data are far from consistent. This is all the more surprising in view of the fact that each determined carnosine by slight modifications of the Koessler and Hanke (7) colorimetric diazo method.

In an early paper, Hunter (5) reported that he found a variable amount of carnosine in different samples of skeletal muscle from animals of the same species, although he did not study discrete muscles. Mitsuda (9) studied the carnosine content of individual muscles of normal and decerebrate cats, and found a variation in the amount of carnosine, both from muscle to muscle, and from animal to animal. He reported values ranging from 341 to 1799 mg. per 100 gm. of muscle, figures which were far higher than Hunter's. An error or misprint in Mitsuda's paper suggests that his carnosine standard may have contained only one-tenth as much carnosine as he stated, in which case his published results would be 10 times too high.

Hunter (6) subsequently made a detailed study of the distribution of carnosine in a number of muscles of the cat, and reported

* A preliminary report of this work was presented before the American Society of Biological Chemists, at Baltimore, April 2, 1938 (13).

that the carnosine content of white muscles was quite variable, both in different muscles of the same cat, and in the same muscle of different cats. He found that the carnosine of white muscle could be decreased by fasting the animal or by preventing use of the muscle, and could be increased by liberal feeding of meat. Hunter found carnosine values which varied from 7 to 494 mg. per 100 gm. of muscle. By contrast, the carnosine of red (soleus) muscle was relatively constant (48 to 90 mg. per 100 gm.) under all conditions studied. Savron (11), in a more recent investigation, found a variation in the carnosine content of biceps muscle of the cat similar to that reported by Hunter. It is worth while to note that Eggleton and Eggleton (4) found a lack of constancy in the carnosine content of various muscles of the frog.

In direct contrast to the results of these investigators, Clifford (1) and Clifford and Mottram (2) observed that all samples of skeletal muscle from any given species contained the same concentration of carnosine. Since these observations are also in conflict with data to be reported in the present paper, we feel that Clifford and Mottram must be in error.

After the development of a method by which anserine could be determined quantitatively, as well as carnosine (see (14)), it was desired to study the distribution of these compounds in various mammalian muscles at our disposal, thereby providing further comparative data concerning both imidazoles. In brief, carnosine was determined by the diazo method after mercury precipitation, and anserine was calculated from the increase in α -amino nitrogen after hydrolysis. The results of our analyses are shown in Table I, where Column *C* represents the total increase in α -amino nitrogen after hydrolysis, Column *B* the increase in α -amino nitrogen to be expected from the hydrolysis of carnosine determined by the diazo method (figures for which are given in Column *E*), and Column *D* ($C-B$) the increase in α -amino nitrogen due to anserine. Anserine (Column *F*) is obtained by multiplying the values in Column *D* by 240/14. The dye standard used in the carnosine determinations was checked against pure carnosine nitrate. The final muscle extracts showed no more than a trace of sulfhydryl by the cyanide-nitroprusside test, and with two exceptions (noted in Table I) gave negative biuret tests.

Owing to the length of time necessary for a complete determination of carnosine and anserine, we usually limited our investigations to two muscles from a given species in any one experiment. Wherever feasible, we chose gastrocnemius (white) and soleus (red) muscles. The absence or rudimentary nature of the soleus muscle in some species did not always make this possible, but where comparison could be made (cat, rabbit, and lion), the concentrations of carnosine and anserine in the soleus were always found to be lower than in gastrocnemius muscle from the same animal.

In view of the confusion in the literature regarding the carnosine of cat muscles, our results for this animal are of interest. In order to obtain sufficient muscle for the anserine determinations, we dissected the gastrocnemius and soleus muscles from nine cats and combined the corresponding muscles (reported as Group I). The same procedure was followed for gastrocnemius and soleus muscles from seven cats (reported as Group II). The carnosine values obtained for soleus are practically identical (47 and 48 mg. per 100 gm. of muscle), and fall within the same range as those reported by Hunter (6) (48 to 90 mg. per 100 gm.). The amount of carnosine in gastrocnemius muscle was found to be greater than in the soleus in both experiments. The anserine figure for gastrocnemius muscle of Group II cannot be considered, as the final extract from this muscle tissue gave a positive biuret test, indicating the presence of higher peptides which would be calculated as anserine in the determination. In Group I, however, more anserine was found in gastrocnemius than in soleus muscles. In fact, the gastrocnemius muscles of the cats of Group I and of the rabbits contained far more anserine than any other muscles studied (478 mg. and 502 mg. per 100 gm. of muscle respectively).

A comparison of the carnosine and anserine content of the gastrocnemius and soleus muscles of the lion with the same muscles of the domestic cat shows that the lion muscles contained less of both compounds. The lions, however, were senile, which may possibly account for the low content of imidazoles.

Muscles from the dog, white rat, and rabbit showed relatively little carnosine. Indeed, Savron (11) has stated that muscles of the dog and white rat fail to give a reaction with the diazo reagent.

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TABLE I
Carnosine and Anserine Content of Various Mammalian Muscles

Animal	Muscle	Amino-N before hydrolysis	Carnosine (diazot)	Increase after hydrolysis	Anserine (C - B)	Carnosine	Anserine
		A	B	C	D	E	F
		Mg. NH ₂ -N per 100 gm. fresh muscle				Mg. per 100 gm.	
Cats, Group I	Gastrocnemius	61.7	13.6	41.4	27.8	220	478
" " I	Soleus	77.3	3.0	21.2	18.2	48	312
" " II	Gastrocnemius	59.2	11.4	53.0	41.6	184	*
" " II	Soleus	50.6	2.9	22.1	19.2	47	329
Lion 1	Gastrocnemius	58.4	3.9	14.9	11.0	62	189
" 2	"	60.0	6.1	19.2	13.1	97	224
" 2	Soleus	71.8	1.4	3.7	2.3	23	39
Dogs	Gastrocnemius	67.5	2.9	15.3	12.4	47	212
Rats	Mixed	53.7	3.0	21.6	18.6	48	319
Rabbits	Gastrocnemius	42.7	5.0	34.3	29.3	80	502
"	Soleus	76.5	3.0	16.0	13.0	48	223
Giraffe	Gastrocnemius	32.2	6.4	20.4	14.0	104	240
Horse	"	46.1	27.0	29.8	2.8	436	48
"	Gluteus	47.0	28.7	29.7	1.0	463	17
"	Soleus					161	
Ox 5	Glossus†	22.0	15.4	17.6	2.2	248	38
" 8	"	26.2	14.1	20.0	5.9	228	101
" 9	"	37.2	15.5	23.9	8.4	250	144
" 10	"	39.8	16.9	24.6	7.7	273	132
" 11	"	45.0	16.9	23.9	7.0	273	120
" 12	"	40.5	17.1	18.7	1.6	276	27
" 15	"	47.4	17.9	23.5	5.6	289	96
" 14	Buccinator	5.2	0.7	0.5	-0.2	12	0
" 6	Shin	17.6	11.8	15.6	3.8	191	65
" 7	Loin	27.8	21.8	29.8	8.0	351	137
" 16	Anterior tibialis	32.8	12.2	19.1	6.9	197	118
" 17	Heart	45.5	0.5	-0.6	-1.1	9	0
" 17	Intestine‡	43.8	1.2	14.1	12.9	19	*

* Anserine value too high, as the presence of polypeptides was indicated by a positive biuret test.

† "Glossus muscle" is used to refer to muscle taken from the genio-glossus, hyoglossus, and styloglossus muscles, without differentiation.

‡ Smooth muscle from intestines.

Protocols

Cats—In Group I the gastrocnemius and soleus muscles were dissected from nine cats which had been used in short time class experiments. The corresponding muscles were combined, and extraction was begun within a few hours after death of the animals. In Group II the gastrocnemius and soleus muscles were dissected from seven cats which had been used for short time class experiments. The corresponding muscles were combined and were placed on ice. Extraction was begun about 15 hours after death of the animals. As the final extract of the gastrocnemius muscles showed a positive biuret reaction, higher peptides were present which led to an erroneously high value for anserine which is not reported.

Lions 1 and 2—Both animals were senile females (Lion 1 about 19 years old, Lion 2 about 15 years old) which were shot by keepers at the Philadelphia Zoo. A portion of the gastrocnemius muscle was dissected from Lion 1, and portions of gastrocnemius and soleus muscles were dissected from Lion 2, by Dr. H. L. Ratcliffe of the Department of Pathology and were placed on ice. Extractions were carried out the following day.

Dogs—The gastrocnemius muscles were dissected from two dogs and were combined. Extraction was begun within a few hours after death of the animals. The soleus muscle is absent in the dog.

Rats—Mixed muscles were dissected from the hind legs of five adult white rats. Extraction was begun within a few hours after death of the animals.

Rabbits—The gastrocnemius and soleus muscles were dissected from five rabbits, and the corresponding muscles were combined. Extraction was begun within a few hours after death of the animals.

Giraffe—The animal died suddenly at the Philadelphia Zoo, apparently from an infection. It had been in a good state of nutrition. A portion of the gastrocnemius muscle was dissected by Dr. Ratcliffe and was placed on ice. Extraction was carried out the following day.

Horse—Portions of the gastrocnemius, gluteus, and soleus muscles were dissected from a horse which was killed at the Veterinary School Hospital because of suspected brain tumor. The quantity of soleus which could be obtained was too small to permit an anserine determination, owing to the rudimentary nature and resultant small size of this muscle in the horse.

Beef Experiments—All samples of ox muscles, with exception of Ox 6 (shin), were obtained at an abattoir within an hour or two after death of the animals. Extractions were begun within a few hours. Glossus muscle was used for most of the experiments because it has little commercial value. Ox 6 (shin) was obtained from a Kosher meat shop, and was said to have come from an animal slaughtered the previous day.

The lowest carnosine figure reported by Savron for any animal was 60 mg. per 100 gm. of muscle, and it may be that he was unable to detect smaller amounts than this.

The horse muscles reported are of interest in regard to the high carnosine and low anserine figures. Unfortunately, no anserine determination could be made on the soleus muscle owing to the rudimentary nature and resultant small size of this muscle in the horse.

Analyses were performed on samples of glossus muscle from freshly slaughtered oxen in order to study the variations which might be encountered in the same muscle of different animals within the species. The carnosine was found to vary from 228 to 289 mg. per 100 gm., whereas the anserine ranged from 27 to 144 mg. per 100 gm. of muscle. The previous nutritional history of the animals is unknown. The variations in anserine in this muscle from animal to animal were relatively greater than the variations in carnosine. We have observed this same circumstance with respect to dog muscles (on which we hope to report in a later paper) and with respect to the buccinator muscle of the ox. In the latter muscle, no anserine was detected in the sample reported in Table I. In other determinations on the same muscle from different oxen (see Table II of the previous paper (14)) the carnosine was 15 and 19 mg. per 100 gm., while the anserine varied from 53 to 87 mg. per 100 gm. of muscle. These low figures may in part be due to the fact that the buccinator muscle is unusually rich in connective tissue which cannot be completely dissected away. Samples of muscle from the shin (foreleg), anterior tibialis, and loin (each taken from a different ox) showed moderate variations in both carnosine and anserine.

Whatever the function of the imidazoles may be in muscle, it might be expected that the total imidazoles (carnosine + anserine) would be more significant than either carnosine or anserine separately. Our data, however, do not indicate any striking constancy for this quantity, either for an individual muscle, or for muscles of a stated species. Thus in the seven experiments on the glossus muscle of the ox, carnosine + anserine varied from 286 to 405 mg. per 100 gm. of muscle. In other skeletal muscles of the ox, the amounts of carnosine + anserine were 256 (shin), 315 (anterior tibialis), and 488 (loin) mg. per 100 gm. of muscle. The highest value for total imidazoles was found in the gastrocnemius muscle of the cats of Group I (698 mg. per 100 gm.). The minimum value for total imidazoles in skeletal muscle was found in the buccinator muscle of the ox (12 mg. per 100 gm.).

It may be seen from Table I, Column A, that the total α -amino nitrogen precipitable by mercuric acetate is quite variable and bears no relation to the amount of either carnosine or anserine in the muscle. Thus, the total amino nitrogen of soleus muscle of Lion 2 is above the average, while carnosine and anserine were quite low. Practically identical figures were obtained for both carnosine and anserine in the soleus muscles of the cats of Groups I and II, although the total amino nitrogen values were quite different. In the series of experiments on glossus muscle from the ox, the variations in total amino nitrogen could not be correlated with variations in carnosine or anserine, or both. We feel, however, that variations in total amino nitrogen may be partially explained by the temperature at which the muscle is extracted, as it has been our experience that extraction at temperatures higher than 70° may lead to an increase in amino nitrogen without corresponding increases in carnosine or anserine.

A single determination was made on the cardiac muscle of the ox, and little carnosine and no demonstrable anserine were found, although the amount of total amino nitrogen was similar to that found in skeletal muscles. An attempt was made to determine carnosine and anserine in a sample of smooth muscle from ox intestines, but the final extract gave a strong biuret test, which indicated that the anserine determination would be too high because of the inclusion of polypeptides. The presence of polypeptides is not surprising in view of the opportunity for proteolysis to occur between the time the intestines were obtained from the abattoir and the time the smooth muscle was ready for hot extraction.

Our analyses indicate that in general a muscle contains far more of one imidazole than the other, and that the same one will be in excess for all skeletal muscles of a given species. Contrary to expectation, anserine appears to be present in higher concentration than carnosine in all mammalian skeletal muscles investigated with the exception of those from the horse and the ox.

In view of the lack of specificity of the quantitative method employed for these analyses, it is worth while to consider other evidence concerning its reliability. The actual existence of carnosine and anserine in muscles for which quantitative figures have been given can best be proved by isolation of the compounds in question. While the isolation procedures give far from quanti-

tative yields, the relative amounts of carnosine and anserine obtained give some indication as to whether they are present in the muscle in high or low concentration. Muscles of the horse and ox have frequently been used for the isolation of carnosine, and it will be seen from Table I that relatively high carnosine concentrations are recorded for most of the muscles studied from these animals. On the other hand, carnosine has been isolated in small amounts only, from muscles of the dog and rabbit, where low concentrations are found by analysis.

Similar relationships are found with anserine. This compound was isolated readily from the muscles of the dog, cat, rabbit, and white rat by Wolff and Wilson (12), from the dog by Parschin (10), and from the rabbit by Deutsch, Eggleton, and Eggleton (3). By analysis, muscles from these animals show large amounts of anserine. Langley and Albrecht (8) reported the isolation of anserine from beef, but made no statement concerning the muscle or muscles used and the amount of anserine found. In connection with the low anserine figures obtained by quantitative determination for skeletal muscles of the horse, it is of interest that anserine was looked for, but not found, in this animal by Wolff and Wilson (12). From a purely analytical point of view, the low anserine values obtained by analysis for certain muscles are perhaps indirect evidence for the absence of compounds which might lead to erroneously high figures for anserine in all muscles.

SUMMARY

Quantitative studies of the distribution of carnosine and anserine in various muscles of the cat, lion, dog, white rat, rabbit, giraffe, horse, and ox are reported. The variations in the carnosine content of muscles of the cat reported by certain investigators have been confirmed. Similar variations in carnosine have been demonstrated in the muscles of other mammals. The amounts of anserine and of total imidazoles (carnosine + anserine) have been found to vary, both in different muscles of an animal, and in the muscles of different members of the species. The amounts of carnosine and anserine in red muscle are lower than those in white muscle from the same animal.

In view of the lack of specificity of the method, it is interesting to note that in animals from which carnosine or anserine, or both,

have been isolated the amounts found by isolation are roughly proportional to the amounts found by quantitative determination.

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THE PROOF OF SYNTHESIS AND THE CONFIGURATIONAL RELATIONSHIPS OF ABRINE*

BY WILLIAM M. CAHILL AND RICHARD W. JACKSON

(From the Department of Biochemistry, Cornell University Medical College, New York City)

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It has been shown by Gordon and Jackson (1935) that the administration of amino-N-methyltryptophane as a supplement to a diet deficient in tryptophane results in prompt resumption of growth by the albino rat.¹ Inasmuch as this derivative of tryptophane had not previously been described, it was found necessary to devise a method for its synthesis. The desired compound was secured by suitable reduction and hydrolysis of 5-(3-indolal)-1-methylhydantoin derived from the condensation of 3-indole aldehyde and 1-methylhydantoin. Shortly following the publication of this synthesis, Hoshino (1935) reported that the crystalline substance, abrine, $C_{12}H_{14}O_2N_2$ (cf. Ghatak and Kaul (1932)) isolated from the seeds of *Abrus precatorius*, L., was an optically active N-methyltryptophane. A critical examination of Hoshino's proof of structure for abrine, and of the method of synthesis and other proof of structure reported by Gordon and Jackson for synthetic *dl*-amino-N-methyltryptophane, scarcely

* A preliminary report was presented at the Thirty-second meeting of the American Society of Biological Chemists at Baltimore, 1938 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. xviii (1938)).

¹ It was postulated (1) that the mechanism for utilization of the methyltryptophane doubtless involves a preliminary demethylation perhaps with the attendant formation of indolepyruvic acid and (2) in consideration of other experiments with sarcosine that the availability of N-methylamino acids might prove to be a rather general phenomenon. Results parallel to the finding for tryptophane have been reported for histidine by Fishman and White (1936) and for methionine and homocystine by Patterson, Dyer, and du Vigneaud (1936). However, Gordon (1937) has found that the corresponding derivative of lysine does not support growth in the absence of this amino acid.

permits of reasonable doubt as to the constitutional identity of the two substances. Nevertheless to establish finally the synthesis of abrine, it remained necessary to make a detailed comparison of abrine and the synthetic compound on a common basis with respect to optical configuration. We have accomplished this by showing that the properties of the racemized abrine and its betaine methyl ester iodide, picrate, and acetyl derivatives are identical with those respectively of the synthetic substance and its corresponding derivatives.

The question of the optical configuration of abrine has also been investigated by quantitative comparison of the maximal optical rotations of the naturally occurring betaine of tryptophane, hypaphorine, and of the betaines secured by exhaustively methylating the natural levo-tryptophane and dextro-abrine.

EXPERIMENTAL

Preparation of Abrine—Abrine was prepared from jequirity seed (*Abrus precatorius*, L.) essentially according to the directions of Hoshino (1935), except that the seed was used without preliminary removal of the scarlet-colored seed coat. 500 gm. of the ground seed were extracted with 2500 cc. of methanol at room temperature over a period of 5 days. The mixture was shaken vigorously two or three times daily. The methanol liquor was filtered off, the seed washed with more methanol, and the combined filtrates and washings evaporated *in vacuo* to a small volume. The concentrated extract was chilled in the refrigerator to yield the crude crystalline material which was filtered off and then washed with ether to remove the contaminating fatty material. This extraction process was repeated four times on each batch of seed, although the material secured from the fourth extraction was generally found to be small in amount and quite difficult to purify. 10 pounds of seed thus yielded 37.8 gm. of impure abrine. Elimination of the gross impurities by recrystallization from boiling water with bone-black treatment reduced the amount of product to 22.7 gm. A final purification carried out in the same manner yielded 21.5 gm. of colorless needles which decomposed at 270–295°, depending on the rate of heating.

$$[\alpha]_D^{25} = +46.0^\circ \text{ (77.7 mg. in 5.0 cc. of 0.5 N HCl)}$$

$$[\alpha]_D^{25} = +62.4^\circ \text{ (49.3 " " 5.0 " " 0.5 " NaOH)}$$

Hoshino reports a specific rotation of 44.4° for the substance in 0.5 N hydrochloric acid at 21° . The calculated analytical values are N 12.85, C 66.02, H 6.47. The values found were N 12.85, C 66.13, H 6.52.

Racemization of Abrine—40 gm. of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ were dissolved in 100 cc. of boiling water in a 500 cc. balloon flask. 5 gm. of abrine were added and the mixture was boiled for a few minutes and then cooled. The optical rotation of the supernatant liquid was determined, and then the mixture was heated in an autoclave at 160° for $4\frac{1}{2}$ hours. Measurement of the optical rotation demonstrated that the compound was 99 per cent racemized after 3 hours and was totally inactive at the end of the $4\frac{1}{2}$ hour period. The mixture was transferred to a large beaker and diluted to about 800 cc. The barium ion was exactly precipitated with dilute sulfuric acid. The combined filtrates and washings from the barium sulfate were concentrated *in vacuo* to about 600 cc., treated with bone-black, filtered, and then further concentrated to a volume of 150 cc. 300 cc. of hot ethanol were added. The solution was further heated and then filtered to remove a small amount of inorganic substance. The filtrate was now concentrated *in vacuo* to a small volume so that the crystals deposited in a heavy mush. A few volumes of ethanol were added and the mixture was allowed to stand in the refrigerator. The 4 gm. of material secured were recrystallized by dissolving in 200 cc. of boiling water, filtering, concentrating to a small volume, and adding an equal amount of hot ethanol. The first crop of crystals weighed 2.8 gm. and a second crop, 0.85 gm. The product consisted of exceedingly fine colorless needles readily soluble in water. In these respects, it closely resembled the synthetic amino-N-methyltryptophane. Natural abrine is much less soluble in water. The analysis of the purified racemized abrine was as follows: N, calculated 12.85; N, found 12.64, 12.58.

Betaine Methyl Ester Iodide—This derivative was prepared by the Engeland method essentially as employed with tryptophane by van Romburgh and Barger (1911). 270 mg. of racemized abrine were dissolved with 50 mg. of sodium hydroxide in 6.5 cc. of hot methanol. 7 cc. of methyl iodide were added and the mixture was refluxed in an oil bath for $8\frac{1}{2}$ hours. 10 mg. of sodium hydroxide dissolved in 0.2 cc. of methanol were added at the end

of each $\frac{1}{2}$ hour interval over the first $7\frac{1}{2}$ hours. The volume was reduced to 4 cc. and the solution allowed to crystallize overnight in the refrigerator. The crystals, washed with methanol and dried, amounted to 200 mg. The material was recrystallized from 5 cc. of boiling water. The colorless methyl ester iodide of the racemic betaine melted with decomposition at 194° .² The analysis for N was 7.21 calculated, 7.29, 7.34 found. An authentic analyzed specimen of the corresponding derivative prepared from the synthetic methyltryptophane (Gordon and Jackson, 1935) melted at 193° . A mixture of the two iodide preparations showed no depression in the melting point.

Monopicate—To 100 mg. of racemized abrine dissolved in 11 cc. of hot water were added 105 mg. (1 molecular equivalent) of picric acid in a saturated aqueous solution (11 cc.). The mixture was placed in the refrigerator overnight. The crystals were filtered off and washed with a little cold water. The 154 mg. of dried product were recrystallized from a mixture of methanol and petroleum ether. The red crystals melted with decomposition at 187° . The analysis was as follows: calculated, C 48.30, H 3.83; found, C 48.46, H 3.89. The authenticated monopicate of the synthetic methyltryptophane (Gordon and Jackson, 1935) melted at 186° with decomposition, and a mixture of the two picrates melted without depression.

Acetyl Derivatives—500 mg. of racemized abrine were dissolved in 2 cc. of 10 per cent sodium hydroxide in a 15 cc. centrifuge tube. A stream of gas from a ketene lamp³ was introduced at the rate of about 6 cc. per minute. This treatment was continued for 1 hour, the solution being kept alkaline to phenolphthalein by the addition of 0.6 cc. of 10 per cent sodium hydroxide every 10 minutes. Three such preparations were combined and acidified with 6 N hydrochloric acid until no more precipitate formed. The precipitated gummy mass was seeded with a few crystals obtained by patient manipulation of the product from a previous preparation. The material was worked with a glass rod to facilitate crystallization, and let stand for 2 hours. It was then filtered

² All melting points are corrected.

³ The authors thank Dr. Max Bergmann of the Rockefeller Institute for his advice on the construction of a platinum filament ketene lamp similar to that used in his laboratory.

off and washed with a little dilute hydrochloric acid. The practically colorless crystals were recrystallized from water to give 1.43 gm. of platelets with a melting point of 171°. Further recrystallization did not result in a change of this melting point.

Analysis— $C_{14}H_{16}N_2O_3$. Calculated. C 64.58, H 6.20, N 10.76
Found. " 64.90, " 6.37, " 10.63, 10.73

25 mg. of the synthetic methyltryptophane (Gordon and Jackson, 1935) were acetylated essentially in the manner described above. The recrystallized product amounted to 17 mg. and melted at 170°. A melting point of a mixture with the acetyl-*dl*-abrine showed no depression.

The acetyl derivative of the natural optically active abrine was also prepared as described for the racemized abrine. Four 500 mg. runs were made. The recrystallized combined product weighed 1.91 gm. and the colorless prisms melted at 175–176°. This melting point was not altered upon one more recrystallization.

$[\alpha]_D^{25} = -148.4^\circ$ (43.3 mg. in 5.0 cc. of 0.1 N NaOH)
Analysis— $C_{14}H_{16}N_2O_3$. Calculated. C 64.58, H 6.20, N 10.76
Found. " 64.62, " 6.24, " 10.68, 10.73

Preparation of Hypaphorine—200 gm. of the seed⁴ of *Erythrina hypaphorus*, Boerl., were crushed and the hulls discarded. The remaining 142 gm. of the seed content were ground, and then let stand with intermittent shaking in 1500 cc. of 95 per cent ethanol at room temperature for 2 days. The seed was filtered off and washed with 200 cc. of the same solvent. The filtrates were concentrated *in vacuo* to a small volume. 200 cc. of water were added and the extract concentrated to 125 cc. The aqueous solution was first freed of fatty material by ether extraction, then treated with sufficient 1.6 N nitric acid to give a deep blue color with Congo red paper, and let stand overnight in the refrigerator. The crystalline hypaphorine nitrate was filtered off, washed with water, and dried. The yield was 8.1 gm. An additional crop of 2.1 gm. was secured by a second extraction. Recrystallization of the total nitrate from 95 per cent ethanol with bone-black

⁴ The authors express their appreciation to Dr. George Barger of the University of Edinburgh for his generosity in furnishing them with a pound of the seed of *Erythrina hypaphorus* as well as suggestions as to the preparation of hypaphorine.

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treatment gave 8.5 gm. of the purified substance with a melting point of about 220°. The nitrate was treated (*cf.* Barger (1914)) with saturated sodium carbonate solution to give the free base, which after crystallization from 95 per cent ethanol amounted to 4.7 gm. The purification with ethanol was repeated to give colorless highly refractive prisms.

Preparation of Optically Active Betaines from Abrine and Tryptophane—5.3 gm. of natural dextro-abrine were dissolved in 125 cc. of methanol containing 1 gm. of sodium hydroxide. 120 cc. of methyl iodide were added and the mixture was refluxed for 4 hours. 5 cc. of methanol containing 0.3 gm. of sodium hydroxide were added at the end of each 20 minute interval over the first 3½ hours. The solution was finally concentrated to 70 cc. and let stand overnight. The crystalline methyl ester iodide was filtered off, recrystallized from hot water, and set aside (2.9 gm.).⁵ The two mother liquors were combined, evaporated to dryness *in vacuo*, and taken up in 50 cc. of hot water. The solution was digested with bone-black and filtered. To the filtrate (81 cc.) were added 9 cc. of 10 per cent sodium hydroxide. The solution was immersed in boiling water for 2 minutes, then cooled, acidified with dilute nitric acid, and let stand in the refrigerator. The salt thus obtained, after recrystallization from water, amounted to 2.7 gm. This was decomposed with 4.6 cc. of 7.6 N (saturated) sodium carbonate. The free base separated largely as an oil which was purified by digestion with bone-black in hot water. The filtrate was evaporated to dryness and the residue was twice crystallized from 95 per cent ethanol to give 0.9 gm. of the pure betaine in the form of prisms.

5.0 gm. of the natural levo-tryptophane subjected to exactly the same procedure yielded 4.5 gm. of the methyl ester iodide⁵ and 0.75 gm. of betaine twice crystallized from 95 per cent ethanol.

DISCUSSION

The experimental data show conclusively that the racemized form of the naturally occurring substance, abrine, is identical with the amino-N-methyltryptophane synthesized by Gordon and Jackson. Of the derivatives employed for comparison of the

⁵ The crystalline methyl ester iodide thus secured was found to be largely racemized and therefore unsuitable for the preparation of the betaine of maximal optical activity.

substance derived from the two different sources, some further mention may be made of the acetyl compound. Ghatak (1933-34) stated that abrine, when refluxed with acetic anhydride and fused sodium acetate, yielded acetyl abrine. The purified substance was described as a microcrystalline powder with a nitrogen content of 10.9 per cent and melting at 286-287°. It was anticipated that Ghatak's preparation could hardly be a monoacetyl derivative of abrine with substitution at the α -amino group because of the extremely high melting point. This proved to be the case, as the monoacetyl compounds of both the optically active and racemic abrine described herewith melt more than 100° lower than does Ghatak's substance.

TABLE I

Comparison of Betaines from Different Sources

The melting point of each substance was 253-254°.

Substance	Nitrogen	$[\alpha]_D^{25}$
	per cent	degrees
Calculated.....	11.38	
Hypaphorine, from <i>Erythrina hypaphorus</i> ...	11.37, 11.45	+113.4*
Betaine, prepared from tryptophane.....	11.14, 11.07	+113.9†
" " " abrine.....	11.27, 11.15	+113.7‡

* 81.2 mg. dissolved in 4.95 cc. of H₂O.

† 54.6 mg. dissolved in 5.00 cc. of H₂O.

‡ 57.4 mg. dissolved in 5.00 cc. of H₂O.

Van Romburgh and Barger (1911) reported that the betaine of tryptophane synthetically prepared from natural levo-tryptophane possesses a positive rotation very close to that of the natural corresponding betaine, hypaphorine.⁶ They showed, therefore, that tryptophane and hypaphorine are of the same configuration. By employing van Romburgh and Barger's procedure, we have shown that abrine belongs to this same configurational series (*cf.* Table I). Inasmuch as tryptophane has been demonstrated by

⁶ Van Romburgh and Barger found $[\alpha]_D = +94.7^\circ$ calculated from the rotation of hypaphorine nitrate prepared from tryptophane. They quoted from Greshoff the rotation of 91-93° for the natural base. Our higher value of $+113.5^\circ \pm 1^\circ$ was uniformly secured in all measurements of the specific rotation of the methylbetaine of tryptophane, irrespective of source.

Lutz and Jirgensons (1931) to possess the configuration of other naturally occurring amino acids, we now have evidence that abrine, like hypaphorine and tryptophane, belongs to the *l* configurational series as now defined and employed for the amino acids occurring in nature (for nomenclature, cf. Freudenberg and Rhino (1924))⁷. The naturally occurring N-methyltryptophane may therefore be designated as *l*(+)-abrine.

SUMMARY

1. Proof of the synthesis of abrine is presented.
2. The monoacetyl derivatives of abrine and racemized abrine have been prepared.
3. Abrine is shown to be of the same configurational series as tryptophane and hypaphorine.

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⁷ It is of interest to note that Gordon (1938) has found that the natural *l*(+)-abrine is practically as effective as tryptophane in promoting growth. The unnatural optical antipode, however, is apparently not thus utilisable.

THE RACEMIZATION OF AMINO ACIDS ON ACETYLA- TION WITH KETENE

BY RICHARD W. JACKSON AND WILLIAM M. CAHILL

(From the Department of Biochemistry, Cornell University Medical College,
New York City)

(Received for publication, July 22, 1938)

In the course of an investigation (1) of the synthesis of abrine, we had occasion to employ ketene as an acetylating agent. Preliminary experiments were made with *l*-tryptophane. In view of the report by du Vigneaud and Sealock (2) that the acetylation of tryptophane with sodium hydroxide and acetic anhydride results in racemization when the latter reagent is in excess, we were led to anticipate the possibility of a similar phenomenon if ketene were employed in place of acetic anhydride. Bergmann and Stern (3) have shown that ketene may be used for the acetylation of a number of amino acids. One of the products prepared by means of this reagent was the optically active acetyl derivative of glutamic acid. Pirie and Hele (4) have similarly synthesized acetyl-*l*-cysteine. However, none of these authors apparently encountered any racemization under their experimental conditions.

Our experiments have shown indeed that ketene acts in much the same fashion as the other anhydride of acetic acid in promoting racemization. When a solution of *l*-tryptophane is kept continuously alkaline to phenolphthalein during the introduction of a stream of ketene gas, the resulting acetyl derivative is not racemized. On the other hand, when the ketene is allowed to generate acetic acid in excess of the alkali present, racemization is found to ensue within an hour. The same results have also been secured in similar experiments with *l*-phenylalanine. It therefore appears reasonable to expect that the racemization with ketene under the stated conditions will prove to be a general reaction for other amino acids. Du Vigneaud and Meyer (5, 6) have shown that several

representative α -amino acids are racemized by acetic anhydride when employed as in the tryptophane experiments already mentioned. They found, however, in further work with proline and N-methylphenylalanine that the presence of an alkyl substituent in the amino group totally prevents the loss of optical activity. To determine whether this limitation is characteristic also of the action of ketene, we attempted the racemization of both proline and abrine.

When proline is subjected to treatment with ketene under conditions promoting the racemization of normal α -amino acids, it is acetylated but not appreciably racemized in the course of 2 hours. Again the result with ketene parallels that reported for acetic anhydride. Most surprising, therefore, was the discovery that abrine, despite its alkylamino group, may be racemized by ketene at a suitable acidity. We regard this observation as quite significant in the consideration of possible mechanisms in the racemization induced by ketene. The racemizing intermediate must, apparently, be of such a nature as to facilitate the transient migration of the hydrogen atom on the asymmetric carbon atom, and attendant racemization. Among intermediates claiming attention is the azlactone. However, an azlactone with the amino nitrogen atom bearing a double bond obviously could not be formed from acetylated abrine in which all three valencies of the nitrogen are engaged with substituent groups. Further investigation of the mechanism involved and its relation to mechanisms of racemization discussed for comparable cases by Bergmann and Zervas (7) and by du Vigneaud and Meyer (6) should prove of interest.

Besides revealing that ketene may be employed to prepare the racemic form of an amino acid from one of its optical antipodes, our experiments have a bearing on the increasing use of this reagent in the search for the effective chemical groups in protein or protein-like products possessing highly specific physiological action. It is conceivable that some of the asymmetric carbon atoms in such substances may be subject to racemization in the manner described, and that at least part of any physiological inactivation following treatment with ketene in an acid solution might be attributable to racemization rather than to the mere blocking of amino groups by acetylation.

EXPERIMENTAL

Acetyl-L-Tryptophane—250 mg. of *L*-tryptophane were dissolved with 2 cc. of 2.5 N NaOH in a 15 cc. centrifuge tube. The gas from the ketene generator was passed in through a capillary tube for 1 hour at a rate of about 6 cc. per minute. The mixture warmed slightly from the heat of reaction. During the hour, 1.5 cc. more of the sodium hydroxide were added in small portions so that the solution was always alkaline to phenolphthalein. The mixture was chilled and acidified with 6 N HCl to give maximum precipitation of the product. Recrystallized from water, it yielded 185 mg. of colorless needles which melted at 188°.¹ The N calculated is 11.38; found, 11.17. $[\alpha]_D^{25} = +30.5^\circ$ (51.8 mg. in 5 cc. of water containing 1 equivalent of NaOH).

Acetyl-dl-Tryptophane—250 mg. of *L*-tryptophane were dissolved in 2 cc. of 2.5 N NaOH, and the experiment conducted as described above except that no extra NaOH was added. In the course of the hour, the mixture became acidic to litmus. The product was recrystallized from water to give 180 mg. of colorless plates which melted at 206°. The nitrogen content was found to be 11.39. The substance was devoid of optical activity.

Acetyl-L-Phenylalanine—200 mg. of *L*-phenylalanine ($[\alpha]_D^{25} = -33.1^\circ$) were dissolved in 2 cc. of N NaOH. Ketene gas was introduced at the rate of about 6 cc. per minute. 2.5 cc. of 2.5 N NaOH solution were added in portions to keep the reaction alkaline to phenolphthalein. The reaction was discontinued at the end of an hour. At this point, the pH was found to be 11, measured by the glass electrode. The mixture was chilled and acidified with 4 N HCl to give 167 mg. of the product. The recrystallized substance melted at 170–171°. $[\alpha]_D^{25} = +47.6^\circ$ (44.9 mg. in 5 cc. of absolute ethanol). This result corroborates the finding of du Vigneaud and Meyer (5) with regard to the sign of rotation of the acetylphenylalanine isomers.

Acetyl-dl-Phenylalanine—200 mg. of the *L*-phenylalanine were dissolved in 2 cc. of N NaOH, and treated with a stream of ketene gas (14 cc. per minute) for 2 hours. The solution became acid to phenolphthalein in about 15 minutes, and the pH at the end of 2 hours was found to be 4. The yield of the crude acetylated product was 141 mg. The substance was recrystallized to give

¹ All melting points reported by us are corrected.

stout needles which were optically inactive. The melting point found of 142–143° is 8° lower than that reported in the literature (cf. (5)). In another experiment, in which the gas was employed at the same rate for only an hour, the recrystallized acetylphenylalanine secured was found to have slight activity: $[\alpha]_D^{25} = +1.2^\circ$.

Racemization of N-Methyltryptophane—250 mg. of abrine ($[\alpha]_D^{25} = +45.7^\circ$ in 0.5 N HCl) were dissolved in 2 cc. of 2.5 N NaOH and treated with ketene gas. The mixture became acid to litmus in $\frac{1}{2}$ hour and the reaction was continued for a total of 2 hours. The pH of the solution was now found to be 3.9. The acetyl derivative was precipitated from the chilled solution with 4 N HCl. After recrystallization from water, the yield was 223 mg. The substance melted at 170–171° and gave $[\alpha]_D^{25} = -1.5^\circ$ (39.1 mg. in 5 cc. of 0.1 N NaOH). The acetyl compound prepared from abrine in an alkaline medium possesses a specific rotation of -148.4° and melts at 175–176°, whereas the product derived from racemized abrine melts at 171° (Cahill and Jackson(1)). The racemization described herewith was, therefore, 95 percent complete. The same result was secured in a second experiment. Our experience leads us to believe that the N-methyltryptophane is more difficultly racemized than tryptophane itself.

Attempted Racemization of Proline—1.28 gm. of proline ($[\alpha]_D^{25} = -82.7^\circ$) dissolved in 10 cc. of 2.5 N NaOH were treated with a rapid stream of ketene gas for 2 hours. The mixture became acid to litmus in 25 minutes, and at the end of 2 hours the pH was 4.5. Dilute sulfuric acid equivalent to the sodium hydroxide employed was now added. The mixture was evaporated *in vacuo* to a low volume, and the product worked up as described by du Vigneaud and Meyer (5). The crude material, crystallized from water and then dried, amounted to 1.3 gm. This, recrystallized from ethanol and ether, yielded 0.64 gm. of prisms which melted at 116–117° and which gave $[\alpha]_D^{25} = -113.2^\circ$ (71.1 mg. in 5 cc. of water). The analysis follows: calculated, C 53.47 and H 7.06; found, C 53.82 and H 7.14. Inasmuch as the specific rotation of our preparation was -113.2° , whereas du Vigneaud and Meyer (6) reported -107.1° for the active compound (0.5 per cent aqueous solution at 27°), it may be concluded that acetylproline is not appreciably racemized by ketene under conditions permitting racemization of the other amino acids studied.

SUMMARY

The treatment of an alkaline solution of tryptophane or phenylalanine with a limited amount of ketene yields the optically active acetamino derivative. If the ketene employed is in excess of the alkali, thus causing the solution to become acidic, these two normal amino acids are rapidly converted to the racemic acetyl compounds.

The presence of an N-alkyl group in the amino acid does not uniformly prevent racemization. No loss of optical activity is observed when proline is treated with ketene in an acid solution, whereas abrine is almost completely racemized.

Racemization is suggested as a possible factor in any loss of physiological activity induced by acetylation of amino groups in specific protein or protein-like substances, when investigated by treatment with ketene in an acid solution.

Addendum—The recent article by Neuberger (8) which has just come to hand reports the racemization of arginine and the partial racemization of histidine through the action of ketene. The author states, "It was noticed in several cases, especially when an excess of alkali was used, that the action of ketone on amino-acids resulted in partial or complete racemization," but makes no mention of the pH of his reaction mixtures at the termination of treatment with ketene. We have detected no racemization with ketene when the aqueous solutions of the amino acids reported by us above were kept continuously alkaline during the treatment with ketene gas. It may also be pointed out that du Vigneaud and associates (2, 5) found no racemization of amino acids with acetic anhydride when the amount of alkali employed was sufficient to neutralize both the acetyl amino acid and the acetic acid produced in the reaction.

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THE ORGANIC ACIDS OF RHUBARB (*RHEUM HYBRIDUM*)*

III. THE BEHAVIOR OF THE ORGANIC ACIDS DURING CULTURE OF EXCISED LEAVES

BY GEORGE W. PUCHER, ALFRED J. WAKEMAN, AND
HUBERT BRADFORD VICKERY

*(From the Biochemical Laboratory of the Connecticut Agricultural Experiment
Station, New Haven)*

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The organic acids of the rhubarb plant have been repeatedly investigated and brief reviews of the literature are to be found in the papers of Culpepper and Caldwell (1) and of Allsopp (2). The latter investigator has recently studied the quantities and distribution of the acids of the plant throughout the year, and has clearly shown that these substances play an active part in the general metabolism, increasing rapidly in both leaves and rhizome during the growing season and undergoing marked changes in the rhizome during the winter.

Our interest in the acids of rhubarb leaves arose from observations upon the behavior of the acids of excised tobacco leaves during culture in water. Although little change takes place when these leaves are exposed to light, in darkness there is a prompt and very extensive loss of malic acid and a corresponding increase in citric acid. Considerable probability was shown to attach to the view that malic acid is transformed into citric acid under these conditions (3). The tobacco plant is a typical representative of the group of neutral plants, as classified by Ruhland and Wetzel; it seemed desirable therefore to study the behavior of the acids in one of the species classified by these investigators as an acid plant (4), especially as the metabolism of the amides and ammonia in the two groups is held to be different, and the or-

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

ganic acids in acid plants are supposed by these investigators to be involved in the protein metabolism.

Composition of Rhubarb Leaves—The organic acid composition of well developed rhubarb leaves (blade and petiole) collected in June of 3 successive years is shown in Table I. There is some variation in detail, as is to be expected since the leaves were derived from field crops grown from old rhizomes under different cultural conditions, but in general nearly 30 per cent of the organic solids consists of organic acids. The last column shows data calculated from Allsopp's analysis of a sample collected in July. This sample appears to have been at about the same stage of

TABLE I

Organic Acid Composition of Rhubarb Leaves (Blade and Petiole)

The figures are given in gm. per kilo of fresh weight of whole leaves.

	June 28, 1935	June 6, 1936	June 7, 1937	Allsopp July 26, 1936
Organic solids.....	68.7	85.8	60.6	
Inorganic solids.....	8.67	10.2	7.04	
Malic acid.....	11.3	14.2	10.5	9.3
Oxalic acid.....	5.2	5.3	4.3	3.6
Citric ".....	1.3	2.0	1.0	1.8
Unknown acid (as malic acid)...	2.2	3.0	1.8	5.1
Total acids.....	20.0	24.5	17.6	19.8
Ratio, $\frac{\text{total acids}}{\text{organic solids}}$	0.291	0.286	0.290	

development as ours, and, save that the unknown acids are somewhat higher, had a similar composition.

About three-quarters of the total amount of organic acids in the leaf is present in the thick and fleshy petiole, and for this reason there is no definite basis for a comparison with the composition of the sessile leaves of tobacco. The stalk of the tobacco plant, unlike the petiole of the rhubarb, contains very little organic acid (5). The tobacco leaf may contain as much as 30 per cent of its organic solids as organic acids, chiefly malic, whereas the blade of the rhubarb leaf, in spite of the higher acidity of the extract, usually contains only from 16 to 18 per cent of the organic solids as organic acids.

EXPERIMENTAL

The leaves employed for the present experiments were those the analysis of which is shown under the dates 1936 and 1937 in Table I. Selection of twenty-leaf samples was made at random from a large lot of leaves pulled from the crown of the plant, care being taken, however, to reject leaves of unusual size or that had been damaged. The 1936 samples (ten in all) weighed 1948 ± 68 gm. each and the 1937 samples (eleven in all) 3481 ± 47 gm., and it is assumed that the chemical composition of the individual samples of each group was constant at the start within a standard error of approximately this relative order of magnitude.

The general methods employed in this laboratory for the culture of excised leaves, and for the determination of the organic acids have been fully described elsewhere (6). It was found necessary to analyze the blade and petiole tissue separately because of the wide difference in behavior. To this end, the blades of each leaf, after removal from the culture solution, were cut with a knife along each of the three main veins so that these remained attached to the petiole. The petiole was then cut into thin slices, both blade and petiole were rapidly dried in a ventilated oven at 80° , and the dried tissue was weighed and ground in a Wiley mill for analysis. The analytical results were calculated in terms of gm., or of milliequivalents in the case of the acids, per kilo of the original fresh weight of the individual sample before culture.

The 1936 leaves were cultured in darkness in distilled water for 261 hours, samples being removed at eight convenient points. The 1937 leaves were cultured under three different conditions: in darkness and in continuous light in water, and in darkness in 0.3 M (5.4 per cent) glucose solution. Samples were taken only at 25, 93, and 165 hours. The conditions of culture are referred to in the following by the letters D and L which indicate culture in darkness and light respectively, and W and G which indicate culture in water or in glucose solution. The year is prefixed to the designation where necessary.

Results

The leaves cultured in darkness in 1936 remained green and turgid for 72 hours, when the first signs of yellowing became ap-

parent at the margins. Chlorophyll breakdown had become fairly extensive in 96 hours and the mesophyll tissue had begun to lose its turgidity. The main veins and the petioles, however, remained turgid nearly to the end of the experiment but the mesophyll had become shriveled and brown in 213 hours. Table II shows the effect upon some of the main components.¹ The water content of both blades and petioles increased temporarily, but the rapid loss

TABLE II

Effects on Rhubarb Leaves of Culture in Water in Darkness

The figures are given in gm. or milliequivalents per kilo of original fresh weight of whole leaves.

Time..	0 hr.	24 hrs.	96 hrs.	165 hrs.	261 hrs.
Water, gm.					
Blade.....	280	295	259	115	18
Petiole.....	624	674	664	644	558
Whole leaf.....	904	969	923	759	576
Organic solids, gm.					
Blade.....	40.0	37.1	33.6	33.8	33.9
Petiole.....	45.8	45.3	41.8	41.0	33.9
Whole leaf.....	85.8	82.4	75.4	74.8	67.8
Total organic acids,* m.eq.					
Blade.....	128	111	105	99	100
Petiole.....	280	290	259	240	179
Whole leaf.....	408	401	364	339	279
Soluble carbohydrates as glucose, gm.					
Blade.....	2.28	1.02	0.81	0.70	1.14
Petiole.....	5.87	4.68	3.00	1.42	1.00
Whole leaf.....	8.15	5.70	3.81	2.12	2.14

* This quantity refers to the total amount of organic acids that can be extracted with ether from dried tissue after acidification with sulfuric acid.

of water from the blades after 96 hours and the retention of water by the petiole and main veins almost to the end are evident. The loss of organic solids due to respiration follows a course in both blade and petiole that can be fairly satisfactorily expressed

¹ A discussion of the changes in certain of the nitrogenous components in these and in the 1937 leaves is given in another paper (7). The complete data with a fuller discussion than is here possible will be presented in a forthcoming bulletin from this Station.

from the full data by straight lines, and it is important to note that the petiole experienced a greater loss than the blade. The loss of a considerable part of the organic acids, especially from the petiole, is in marked contrast to the behavior of the organic acids of tobacco leaves under similar conditions. The loss was accompanied by a gradual change in the reaction of the tissues, the extract from the blade changing from pH 4.0 to 5.5 and that from the petiole from pH 3.0 to 4.0 during the period of study.

The soluble carbohydrates diminished rapidly, particularly in the blade, but comparison of the change with that of the organic

TABLE III
Organic Acid Composition of Rhubarb Leaves during Culture in Water in Darkness

The figures are given in milliequivalents per kilo of original fresh weight of whole leaves.

	0 hr.	24 hrs.	96 hrs.	165 hrs.	261 hrs.	0 hr.	24 hrs.	96 hrs.	165 hrs.	261 hrs.
	Malic acid					Oxalic acid				
Blade.....	30	23	23	22	21	43	40	44	40	40
Petiole.....	182	175	163	137	85	77	71	75	75	71
Whole leaf.....	212	198	186	159	106	120	111	119	115	111
	Citric acid					Unknown acids				
Blade.....	17.9	17.5	13.3	12.0	10.8	36	30	28	25	28
Petiole.....	12.8	12.5	13.4	12.3	8.8	8	32	19	15	15
Whole leaf.....	30.7	30.0	26.7	24.3	19.6	44	62	47	40	43

solids shows that only about one-third of the loss from the whole leaves can be accounted for as respiration of soluble carbohydrates.

Table III shows the changes in the individual organic acids of both blade and petiole. The malic acid of the blade diminished by only 9 milliequivalents, whether by transport to the petiole or by chemical change is not apparent, but the malic acid in the petiole diminished by 97 milliequivalents or approximately 6.5 gm. The change for the whole leaf represents the loss of about half the malic acid present.

If the losses of 4.9 gm. of soluble carbohydrates and 6.5 gm. of malic acid from the petiole are combined, the total agrees very

closely indeed with the loss of 11.9 gm. of organic solids. Furthermore, the loss of total organic acids from the petiole was 101 milliequivalents and it seems reasonably certain therefore that the malic acid which disappeared from the petiole was completely oxidized to volatile products. A large part of the malic acid of tobacco leaves likewise disappeared under similar circumstances, but in this case there was a corresponding and chemically equivalent increase in citric acid. The evidence pointed clearly to a transformation of malic into citric acid in the tobacco leaf but in the rhubarb leaf, at least in the petiole, malic acid appears to be largely respired during culture in darkness, sharing this fate with the soluble carbohydrates.

Compared with the extensive change in the malic acid, the changes in the other acids are of minor importance. Citric acid was present only in small amounts at the start, and about one-third of it slowly disappeared. The detailed data for oxalic acid fluctuate above and below a mean figure in a manner that suggests that no significant change occurred in either blade or petiole. The unknown acids showed a significant temporary increase in the petiole in the early stage of culture but no other change was detected. During culture in water in darkness, therefore, the chief metabolic change in the organic acids of rhubarb leaves is the respiration of a large part of the malic acid, mostly from the petiole.

A summary of the results of the determinations of the organic acids in the leaves studied in 1937 is shown in Table IV. The total organic acid content and the malic acid content of both blade and petiole are given, but only the whole leaf data for the other components are included, in view of the minor nature of the changes. The hydrogen ion activity of the blade changed from pH 4.0 to 4.8 in the DW and DG experiments but only to pH 4.3 in the LW series. The petioles in all cases changed only from pH 3.0 to 3.2 in the period studied.

The general behavior of the DW leaves resembled that noted in the 1936 experiment but, in both LW and DG leaves, chlorophyll breakdown had not become at all serious at the end of 93 hours and the samples were still in far better condition than the DW leaves at the end of 165 hours. The petioles and main veins remained turgid throughout.

The total organic acids in the DW series had apparently changed very little at the end of the culture period, unlike that of the leaves examined in 1936, but the amount present had dropped materially in 93 hours. A continuous loss was observed through a series of successive samples in the 1936 experiment.

The behavior of the organic acids of the petioles was markedly different in the three experiments. In the DW series, the total

TABLE IV
Changes in Organic Acids of Rhubarb Leaves under Different Conditions of Culture

The figures are given in milliequivalents per kilo of original fresh weight of whole leaves.

Culture conditions*	DW				DG			LW		
Time.....	0 hr.	25 hrs.	93 hrs.	165 hrs.	25 hrs.	93 hrs.	165 hrs.	25 hrs.	93 hrs.	165 hrs.
Total organic acids										
Blade.....	73	74	61	72	69	66	60	68	64	60
Petiole.....	223	219	219	213	229	257	254	243	240	225
Whole leaf.....	296	293	280	285	298	323	314	311	304	285
Malic acid										
Blade.....	16	11	8	9	14	10	7	13	13	10
Petiole.....	140	140	134	116	153	160	142	164	122	103
Whole leaf.....	156	151	142	125	167	170	149	177	135	113
Citric acid										
Whole leaf.....	15	14	12	11	15	15	14	13	14	12
Oxalic acid										
Whole leaf.....	97	104	96	102	91	100	97	95	94	92
Unknown acids										
Whole leaf.....	27	26	30	46	26	38	55	26	61	67

* The letters D and L indicate culture in darkness and light respectively; the letters W and G indicate culture in water or in 0.3 M (5.4 per cent) glucose solution.

acids fell slightly but in the DG series, slowly increased. In the LW series, the increase was prompt, but later the acids decreased to about the initial level.

These changes become intelligible in the light of the behavior of the malic acid. In the blades in all cases, about half the small amount originally present disappeared, though whether by transport to the petiole or by chemical change is not evident. In the

petioles of the DW series there was a considerable loss, in the DG series a definite although slow gain followed by a slow loss, and in the LW series there was an immediate gain followed by a rapid loss, so that the final value was far below the initial value. Examination of the figures for the whole leaf shows that these changes were all materially in excess of those that could have been produced by transport from the blade.

The prompt increase in the malic acid in the petioles during culture in light at once raises the question of its origin. The increase in 25 hours was 24 milliequivalents or 1.5 gm. During the same interval, the whole leaves lost 4 gm. of organic solids, including 1.3 gm. of soluble carbohydrates, and gained 15 milliequivalents of total organic acidity. Thus the newly formed malic acid must have arisen from a precursor which was not an acid, and there was insufficient soluble carbohydrate originally present to account for all of it. The inference is clear that malic acid was formed, at least in part, either directly or indirectly from the products of photosynthesis, and, accordingly, malic acid metabolism is definitely linked with the carbohydrate metabolism. This conclusion is supported by the result of the culture in darkness in glucose solution. The contrast between the data for the petioles in the DW and DG experiments makes the specific influence of glucose clear. The increase of 20 milliequivalents of malic acid (1.3 gm.) in 93 hours of culture in glucose may be compared with the increase of 34 milliequivalents of total organic acids and of 10 milliequivalents of unknown acids. The newly formed malic acid must therefore have been derived from some non-acidic substance. Data for glucose absorption, obtained by analysis of the culture solutions employed, indicated that 11.4 gm. of glucose were taken up in 93 hours per kilo of whole leaf. The organic solids of the whole leaves increased only 2 gm. in this period, and the soluble reducing sugar content increased by only 0.5 gm. It is therefore clear that the absorbed glucose was rapidly metabolized, the greater part being respired.

Whether a part of it was directly converted into malic acid, or whether the presence of a large supply of glucose permitted the leaves to synthesize malic acid from components that would otherwise have had some other fate cannot be decided; but there is a definite analogy between the behavior of the malic acid in the

leaves cultured in glucose solution in darkness and in the leaves cultured in water in light, which were presumably able to produce carbohydrates by photosynthesis. This analogy was most striking in the early phase of the experiment when the leaves were in good condition and absorption of glucose solution on the one hand, and photosynthesis on the other, were most active.

The citric acid diminished in all three experiments, but the change was significant only in the DW series. The detailed data for blade and petiole show evidences of transport from blade to petiole in each case, particularly in the LW series. In the presence of glucose, the apparent loss of total citric acid was no greater than the error of the determination; in light it was just appreciable. Citric acid plays a very minor rôle in the metabolism of the rhubarb leaf, in marked contrast to its behavior in tobacco leaves.

The oxalic acid determinations in the DW and DG experiments show no evidence of transport of oxalic acid from blade to petiole, but transport of a little apparently took place in light. The data for the whole leaf suggest that at most a very small change occurred in the quantity present save possibly in the LW series where there may have been a slight loss. The acids of the unknown group, calculated by difference, increased in all cases, the change being most important in the LW series. The total amount present, however, is small in relation to the malic acid, and no interpretation of the behavior can be made save to point out that an increase in this group of substances is perhaps to be anticipated under conditions of rapid carbohydrate metabolism. The protein of the leaves in the DW experiment underwent extensive digestion in the interval between 25 and 93 hours, but there was little or no effect upon the group of unknown acids in this period and, accordingly, it may be inferred that these substances are not to any great extent concerned with the protein metabolism.

DISCUSSION

The chief effect upon the organic acids of the culture of rhubarb leaves either in light or darkness is upon the malic acid. In darkness this diminishes; in light it increases, at least temporarily. The effect of culture in light can be simulated by culture in darkness in glucose solution. There is therefore a definite relation-

ship between malic acid and the carbohydrate metabolism. Malic acid arises under conditions of adequate carbohydrate supply, and disappears under conditions where the carbohydrates are otherwise utilized. Whether or not the newly formed malic acid is derived from transformations of the carbohydrate does not appear. It is possible that the glucose so stimulated the general metabolism that malic acid was produced from some other source, but light energy plays no essential part in the reaction and malic acid is therefore not a direct product of photosynthesis.

Speculation upon the details of the intermediary metabolism of the malic acid in the rhubarb leaf at the present time is hardly justified. The numerous suggestions in the literature have been carefully reviewed and compared by Bennet-Clark (8-10), but few of them are supported by cogent evidence and none appears to have general validity. The success of our attempt to influence the malic acid content by culture in glucose solution suggests a possible approach to a more detailed study of the phenomenon.

The general effect upon the acids of rhubarb leaves of culture in light is a confirmation of results obtained many years ago by Steinmann (11) who recorded an increase in the titratable acidity during the day and interpreted this as a possible indication of the direct photosynthesis of organic acids. The increase of 9 per cent of the morning acidity he observed is of the same order of magnitude as the increase in malic acid we have found during 25 hours of culture in continuous light.

The demonstration that malic acid may arise during culture in glucose solution in darkness is of particular importance in connection with the views of Kostytchev and Tschesnokow (12), and of Ruhland and Wetzels who hold that the plant acids are derived from the metabolism of protein. The latter investigators maintain that this is specifically the case in the rhubarb plant, and consider that one of the functions of the newly formed organic acid is to neutralize the ammonia that also arises from the oxidation of the amino acids, thereby rendering it harmless to the plant. If this were the case, one would expect parallel increases in both organic acids and ammonia during culture, whereas in fact there is a decrease in organic acids during the period when ammonia is being most rapidly formed. To what extent observations upon

the culture of excised leaves can be applied to the explanation of the metabolism of *normal* leaves is, of course, debatable, but the evidence is clear that the metabolism of malic acid in the rhubarb leaf is intimately connected with that of the carbohydrates.

It is probably desirable to add a word on the danger of generalization from the few facts regarding the metabolism of malic acid that have been ascertained. The behavior in rhubarb is entirely different from that in the tobacco plant. The only other case in which any substantial body of evidence has been accumulated is that of the acid metabolism in succulent plants such as *Sedum*, *Crassula*, *Bryophyllum*, and *Opuntia* species. In these, organic acid appears during culture in darkness and disappears in light, the exact reverse of the behavior in rhubarb. Mayer many years ago showed that oxygen is liberated during the disappearance of the acid, and obtained considerable evidence that malic acid is the substance involved in the changes (13). This phenomenon seems indeed to be common and Bennet-Clark (8) gives a list of numerous species in which it has been noted. On the other hand, specific methods for the identification of the acid concerned have been applied in few cases. There is a large literature devoted to the discussion of the problem (9), but no well established theoretical interpretation has yet been found although it seems clear that in this case also malic acid arises from carbohydrate sources.

SUMMARY

The organic acids of rhubarb leaves diminish during culture in water in darkness, and evidence is presented that the change is due to respiration of a large part of the malic acid in the petiole.

Culture in water in light brings about a temporary increase in the organic acids in the petiole which is shown to be due to an increase in malic acid. Culture in glucose solution in darkness brings about a similar but more prolonged and even more extensive change, and the evidence is clear that the newly formed malic acid arises directly or indirectly from the carbohydrate.

The organic acid metabolism of rhubarb leaves differs fundamentally from that of tobacco leaves during culture under similar conditions, and the behavior of the malic acid in both of these

species differs from its behavior in succulent plants. Accordingly generalizations upon the chemical relationships of malic acid in green leaves are difficult to frame.

Neither citric nor oxalic acid appears to be extensively involved in the chemical changes of the organic acids of excised rhubarb leaves during culture, and the acids of the so called unknown group likewise play a minor rôle. No evidence was found to link any of these acids with the protein metabolism.

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MODIFICATION OF THE METHOD OF SHOHL AND BENNETT FOR THE DETERMINATION OF POTASSIUM IN SERUM AND URINE*

BY W. V. CONSOLAZIO AND JOHN H. TALBOTT

(From the Medical Clinic of the Massachusetts General Hospital and the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)

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The Lindo-Gladding procedure (1) as adapted by Shohl and Bennett (2) for determination of potassium in small amounts of biological material has been modified. The entire procedure may be carried out without transfer in one container, a cone-pointed quartz tube. The final titration is performed with a modified Rehberg microburette (3) with gain in rapidity and facility (4).

Reagents and Apparatus—

Platinum chloride, 20 per cent solution in N HCl.

Absolute ethyl alcohol, saturated with potassium chloroplatinate.

Sulfuric acid, 50 per cent by volume.

Potassium iodide, 2 N, iodate-free.

Sodium sulfate, 50 per cent saturated, potassium-free.

Cone-pointed quartz tube, 10 cc. capacity.¹

Rehberg microburette, 0.225 cc. capacity.²

Glass stirring rods, 1.0 mm. by 150 mm.

Procedure

An amount of serum or urine which contains from 0.002 to 0.01 milliequivalent of potassium is transferred to a cone-pointed quartz tube. Convenient quantities are 1 cc. of serum and 0.2 cc. of urine. Naturally occurring phosphates and iron do not

* This investigation was aided by the Corn Industries Research Foundation.

¹ Macalaster-Bicknell Company, No. C-1546, 1937 catalogue.

² Macalaster-Bicknell Company, No. C-1535, 1937 catalogue.

interfere and need not be removed. 1 drop of 50 per cent sulfuric acid is added. The mixture is placed in an oven at 110° for 12 hours or until charring occurs. In urine or other material which contains less than 0.10 milliequivalent of sodium in the sample, 1 drop of 50 per cent saturated sodium sulfate is added before charring. The tube is placed in a cold electric muffle furnace and ashed overnight at a temperature between 450 – 500° . The temperature should be raised slowly to prevent spattering. The tube is then cooled to room temperature and 0.2 cc. of 20 per cent platinum chloride in N HCl is added. The bottom of the tube is agitated vigorously. The sample is next treated with 5 cc. of absolute alcohol which has been saturated with potassium chloroplatinate and filtered before use. The mixture is stirred for several seconds with a fine glass rod to insure complete precipitation of the potassium chloroplatinate. The rod is rinsed with 1 cc. of filtered alcohol and the tube is placed in a rack for 30 minutes.

The tube is centrifuged at approximately 2000 R.P.M. for 5 minutes. The supernatant fluid is removed by suction through a glass tube drawn to a fine capillary. (The capillary prevents a back flow of mother liquor which might disturb the precipitate.) The precipitate is then washed with 5 cc. of filtered alcohol, and the mass broken up by a fine glass rod and dispersed throughout the wash solution. The rod is rinsed a second time with 1 cc. of alcohol and the process of centrifuging and removal of the supernatant fluid is repeated.

The tube is placed in a water bath at a temperature of about 70° . The temperature is slowly raised to boiling and the alcohol completely evaporated. (If the tube is placed in boiling water, the alcohol may spatter and some precipitate is lost.) The precipitate is covered with 5 cc. of distilled water, the tube remaining in the water bath at about 80° until solution of the potassium chloroplatinate is complete. Agitating the tube aids the solution. Approximately 1 cc. of $2N$ potassium iodide is added and the temperature maintained for a few minutes to insure complete formation of the iodoplatinate. The tube is cooled to room temperature by tap water. The solution is titrated finally with $0.1N$ sodium thiosulfate delivered from a Rehberg burette. The end-point is a clear lemon-yellow color, free from red. 1 drop of thiosulfate is ejected from the burette and the tip wiped with filter paper,

before refilling. Rewiping before the first titration is necessary; further wiping between titrations is not necessary.

As an alternate procedure the iodoplatinate may be determined colorimetrically.

Comment

Quartz tubes are preferred to Pyrex tubes because of the higher yield. The lower yield (about 5 per cent) in Pyrex tubes may be caused by the fusion of potassium salts to the glass.

It is realized that the temperature at which potassium salts are ashed affects their percentile recovery. Known quantities of potassium chloride were ashed with sulfuric acid at a temperature of 410° for 40 hours with an 8 per cent loss. 2 gm. of potassium chloride were treated with sulfuric acid and ignited in a platinum crucible. There was a significant loss of salt due to creeping. Potassium sulfate behaved similarly. If, however, sodium sulfate was added to the salt before charring began, other conditions remaining equal, the loss was less than 2 per cent. The use of sodium sulfate enables one to maintain an ashing temperature of 500° for more than 12 hours with only negligible loss of potassium. It is supposed that the sulfates of sodium and also of calcium and magnesium act as occluding agents and favor precipitation of potassium chloroplatinate.

The evaporation of alcohol has replaced the washing of the precipitate by a potassium chloride solution saturated with potassium chloroplatinate as recommended by Shohl and Bennett. If the precipitate is washed with a solution of potassium chloride, the recovery is from 5 to 10 per cent low.

Recovery of potassium from a known solution is about 50 per cent if 80 per cent alcohol (5) is used in place of absolute alcohol. A concentration of 95 per cent alcohol gives a recovery of about 95 per cent. In order to maintain the final concentration of alcohol close to 100 per cent, 0.2 cc. of a combined solution of concentrated platinum chloride and hydrochloric acid may be added. The yield with this solution is 98 per cent or more. Although we have suggested absolute alcohol saturated with potassium chloroplatinate for precipitation, this is not necessary, as absolute alcohol alone gives an error of less than 0.5 per cent.

A black ring of precipitated platinum around the wall of the

tube may be observed occasionally during titration. When this is noted it indicates that all of the alcohol has not been evaporated and some of the iodoplatinate has been reduced to platinum. Such analyses should be discarded and the tubes cleaned with aqua regia.

Titration of the iodoplatinate while hot is inadvisable with a Rehberg burette. Sufficient heat radiates from the tube to cause some expansion of the titrating fluid. A sharper end-point is obtained and the color change is stable if the solution is at room temperature.

Results

In Table I are presented data from sixteen consecutive analyses of a known solution which contained 5.00 milliequivalents per liter of potassium in addition to other salts found in serum. The average recovery was 5.00 milliequivalents per liter. The maximum error was ± 2 per cent. This is greater accuracy than we have been able to obtain with the method of Shohl and Bennett.

In Table II, are presented data from eleven samples of human sera. 1 cc. samples with the method described are compared with 10 cc. or 20 cc. with the Lindo-Gladding gravimetric method. The agreement was better when 20 cc. samples were used in the macro-method. In six of the sera the maximum deviation was less than 5 per cent. In the remainder the deviation varied between 5 and 10 per cent. There was no consistent difference between the volumetric and gravimetric methods. It is admitted that these results are not ideal but it is probably as fallacious to implicate the titrimetric as the gravimetric method. The gravimetric procedure is more difficult and the opportunity for technical errors is probably greater, owing to loss of potassium salts during ashing in open dishes. Under such conditions the greater loss takes place within the first few hours. Following this a constant weight is reached and ignition at 750° for 12 hours results in no appreciable change in weight.

Known amounts of potassium chloride were added to distilled water and ox serum and the resulting concentrations determined by the micromethod and the macromethod of the Association of Official Agricultural Chemists (1). These observations are given in Table III. The ox serum was found by repeated determinations

TABLE I

Recovery of Potassium from Known Solution

The solution contained the following constituents: sodium 140.0, potassium 5.0, calcium 5.0, magnesium 2.0 milliequivalents per liter.

Sample No.	Method described
	<i>m.eq. per l.</i>
1	4.94
2	4.99
3	4.98
4	4.97
5	4.97
6	4.97
7	5.10
8	5.06
9	4.93
10	4.91
11	5.02
12	5.00
13	4.99
14	4.95
15	5.05
16	5.12
Average.....	5.00

TABLE II

Concentration of Potassium of Human Sera

Subject	Lindo-Gladding method	Method described (1 cc. samples)
	<i>m.eq. per l.</i>	<i>m.eq. per l.</i>
J	4.81 (10 cc. samples)	4.55
A	4.42 (10 " ")	4.32
P	4.39 (10 " ")	3.98
W	4.16 (10 " ")	4.06
S	3.98 (10 " ")	4.12
K	4.30 (10 " ")	4.01
C	3.74 (10 " ")	3.95
P	5.66 (20 " ")	5.58
T	7.07 (20 " ")	7.11
C	5.71 (20 " ")	6.17
W	5.91 (20 " ")	5.98

to contain exactly 5.00 milliequivalents per liter of potassium. The recovery from the aqueous solution by the micromethod was 0.07 and 0.13 milliequivalent per liter less than the theoretical. By the macromethod the recovery from the same solutions was 0.45 and 0.33 milliequivalent, respectively, less. By the micro-technique recovery of potassium added to the ox serum was better

TABLE III

Recovery of Potassium Added to Distilled Water and to Ox Serum

Sample No.	Vehicle	Theory	Method described (1 cc. samples)	Association of Official Agricultural Chemists method (10 cc. samples)
		<i>m.eq. per l.</i>	<i>m.eq. per l.</i>	<i>m.eq. per l.</i>
1	Distilled water	6.67	6.60	6.22
2	“ “	2.65	2.52	2.32
3	Ox serum	5.00	5.00	4.53
4	“ “	5.21	5.11	4.77
5	“ “	5.53	5.43	5.21
6	“ “	7.32	7.25	6.85

TABLE IV

Concentration of Potassium in Human Urine

Sample No.	Lindo-Gladding method (10 cc. samples)	Method described (0.2 cc. samples)
	<i>m.eq. per l.</i>	<i>m.eq. per l.</i>
1	25.6	25.4
2	21.8	19.3
3	27.5	26.6
4	23.8	21.6
5	20.9	21.3
6	23.9	23.0
7	13.0	13.0

than 98 per cent. By the macrotechnique, recovery varied between 88 and 92 per cent.

In Table IV data are presented from seven samples of urine. In five samples the variation between the titration and Lindo-Gladding method was less than 4 per cent. The average deviation was 3.5 per cent.

SUMMARY

A modification of the Shohl and Bennett method for determination of potassium in biological material is given. The entire procedure is carried out in a cone-pointed quartz tube and the titration fluid is delivered from a Rehberg microburette. The average error is believed to be less than 2 per cent.

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STRUCTURE OF DESOXYRIBONUCLEIC ACID

ON THE DIPHOSPHORIC ESTERS OF PYRIMIDINE- DESOXYRIBOSIDES

By P. A. LEVENE

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

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There appeared recently an article by Bredereck and Caro¹ dealing with the structure of desoxyribonucleic acid. The authors claim to have isolated on mild hydrolysis only the *monophosphoric* acid and not the diphosphoric esters of the two pyrimidine nucleosides, as had been previously reported from our laboratory.^{2, 3, 4}

It is expected from a critic of the results of an earlier investigation that his arguments should be based on more rigorous evidence, particularly when an interval of 20 years separates the two investigations. A scrutiny of the experimental evidence presented by Bredereck and Caro reveals that it would scarcely have been considered rigorous even at the date of the earlier publications.

In particular, Bredereck and Caro base their conclusions on the analysis of brucine salts and of amorphous barium salts which are identified by their barium content only. Not a single complete analysis of a barium salt is given. We convinced ourselves long ago that such data deserve little reliance or credence.

Had Bredereck and Caro accurately repeated our experiments, they should have had no difficulty in obtaining a crystalline barium salt of the diphosphoric ester of the thyminedesoxyriboside, for it crystallizes without much difficulty.

A complete analysis of a crystalline barium salt deserves, in our judgment, more credence than the mere barium estimation of

¹ Bredereck, H., and Caro, G., *Z. physiol. Chem.*, **253**, 170 (1938).

² Levene, P. A., and Mandel, J. A., *Ber. chem. Ges.*, **41**, 1905 (1908).

³ Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, **12**, 411 (1912).

⁴ Levene, P. A., *J. Biol. Chem.*, **48**, 119 (1921).

an amorphous salt, or even than a complete analysis of a brucine salt. Indeed, the elementary composition of the brucine salt of the monoester does not differ sufficiently from that of a diester to permit a final decision between the two structures. The brucine salts present a great advantage for the purpose of preliminary fractionation but not for identification.

Realizing the importance of the claims of Bredereck and Caro on the theory of structure of the desoxyribonucleic acid, we re-analyzed one of the substances prepared by us in 1920-21; namely, the barium salt diphosphoric ester of cytosinedesoxyriboside. The salt had remained a perfectly white powder, soluble in water. The composition of the substance had remained practically unchanged.

Unfortunately, the old sample of the barium salt of the diphosphoric ester of the thyminedesoxyriboside could not be found. However, several gm. of a brucine salt labeled "crude brucine salt of hexothymidine diphosphoric acid" were found. From this the barium salt of the diphosphoric ester was obtained in pure state. The brucine salt was recrystallized from 35 per cent alcohol four times, the salt then acquiring the appearance of small elongated prisms. The brucine salt was converted into the lead salt, and that into the barium salt. The composition of this substance was in perfect agreement with the theory for the barium salt of the diphosphoric ester.

The history of the monoester of the pyrimidinedesoxyribosides is the following: In 1908 Levene and Mandel² described a substance having the composition of the monophosphoric ester of thyminenucleoside. In 1912 Levene and Jacobs³ again obtained substances having the composition of the monophosphoric ester of pyrimidine nucleosides as well as some of the diphosphoric esters. In 1920-21 Levene⁴ obtained the barium salts of the diphosphopyrimidinedesoxyribosides and also a barium salt having the nitrogen to phosphorus ratio of a monophosphoric ester of a pyrimidine nucleotide. On further purification the latter was found to consist of the diphosphoric ester. At that time no monophosphoric ester was isolated from the products of hydrolysis. That the monophosphoric ester might have been missed in the course of the many fractionations leading to the preparation of the diphosphoric esters is not impossible and was admitted in our

publications of 1920-21. For the theory of the structure of desoxyribonucleic acids, the monophosphoric ester is of lesser importance. On the other hand, it is peculiar that as our experience in handling nucleotides has increased, we have found that the crude barium salt which contained the barium to phosphorus ratio corresponding to the monoester on purification yielded in the main the diphosphoric ester. Incidentally, Bredereck and Caro admit that some of their fractions might have contained the diphosphoric acids.

EXPERIMENTAL

The barium salt of the diphosphocytidinedesoxyriboside analyzed in 1920-21 was reexamined. It was completely soluble in water. The solution did not form even a turbidity with a solution of barium hydroxide and gave no test for free phosphoric acid with an ammonium molybdate solution.

3.905 mg. substance: 2.390 mg. CO_2 and 0.612 mg. H_2O

5.700 " " : 36.495 " $\text{Mg}_2\text{P}_2\text{O}_7$

7.150 " " : 0.391 cc. N_2 at 28° , p 756 mm.

$\text{C}_9\text{H}_{11}\text{N}_3\text{P}_2\text{O}_{16}\text{Ba}_2$. Calculated. C 16.41, H 1.68, N 6.38, P 9.43

Found (1920-21). " 17.05, " 2.12, " 6.17, " 9.40

" (1938). " 16.69, " 1.73, " 6.17, " 9.29

The better agreement of the present values with the theory is due to the fact that the older values were obtained by the macromethods.

Barium Salt of Diphosphothyminedesoxyriboside—The crude brucine salt prepared in 1920-21 was recrystallized four times from 35 per cent ethanol. The salt then acquired the shape of small elongated flat prisms.

4.070 mg. substance: 8.100 mg. $\text{Mg}_2\text{P}_2\text{O}_7$

5.080 " " : 2.97 cc. N_2 at 31.5° , p 752.5 mm.

$\text{C}_{102}\text{H}_{121}\text{O}_{27}\text{N}_{16}\text{P}_2 + 14\text{H}_2\text{O}$. Calculated. N 6.27, P 2.82

Found. " 6.48, " 2.89

The above salt was decomposed by means of aqueous ammonia. The aqueous solution was concentrated to small volume, acidulated, and treated with a solution of neutral lead acetate. The lead salt was thoroughly washed with water and was decomposed in the usual way, and the filtrate from the lead sulfide was rendered

faintly alkaline to phenolphthalein. A few floccules of barium phosphate were removed. On concentration under reduced pressure at room temperature a granular precipitate consisting of striated globules soon began forming. Dried at 100° and at a pressure of 400 mm., the substance had the following composition.

11.420 mg. substance:	17.68 mg. CO ₂ and 1.905 mg. H ₂ O
4.005 " " :	25.300 " Mg ₂ P ₂ O ₇
7.975 " " :	2.97 cc. N at 28.5°, <i>p</i> 762 mm.
(C ₁₀ H ₁₂ N ₂ O ₁₁ P ₂ Ba ₂). Calculated.	C 17.83, H 1.81, N 4.16, P 9.22
Found (1920-21).	" 18.11, " 1.93, " 4.04, " 8.97
" (1938).	" 17.68, " 1.86, " 4.13, " 9.17

The mother liquor was further concentrated and poured into alcohol, giving a second precipitate.

THE ERGOT ALKALOIDS*

XVI. FURTHER STUDIES OF THE SYNTHESIS OF SUBSTANCES RELATED TO LYSERGIC ACID

BY WALTER A. JACOBS AND R. GORDON GOULD, JR.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

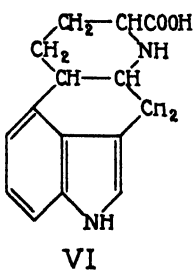
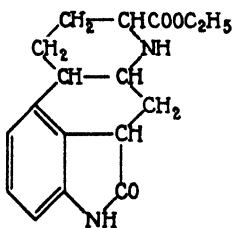
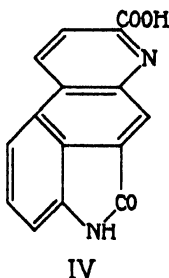
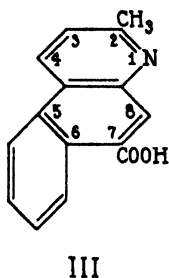
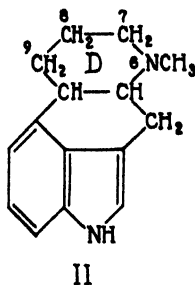
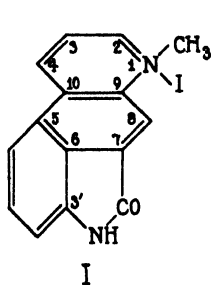
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In a previous communication (1) we have described the synthesis of ergoline, a substance consisting of an unsubstituted, tetracyclic indole nucleus which is at the same time a derivative of a decahydroquinoline and is the ring system that has been derived for lysergic acid (2). Attempts have since been made to extend the program of synthesis to include the substituting groups present in lysergic acid; namely, the N-methyl group in Ring D and the carboxyl group which had been assumed to be in position 7 in Ring D. However, in view of the recent evidence, which we have presented elsewhere (3, 4), that the carboxyl group of lysergic acid is in position 8, it has now become more urgent to extend the study in this direction. We wish at this point to record our experience in the attempt to introduce a carboxyl group only in position 7.

It has been possible by the following series of steps to synthesize 6-methyl ergoline (Formula II). In order to avoid the formation of a mixture of methylated derivatives which would be expected if methylation were attempted after reduction to the polyhydroquinoline stage, methylation was directly accomplished with the quinoline derivative itself. Thus the lactam of 3'-amino-5, 6-benzoquinoline-7-carboxylic acid (1) gave smoothly the quinolinium methiodide (Formula I). This salt on catalytic hydrogenation gave the hydroiodide of the 1-methyl-2,3,4-trihydroquinoline derivative which was studied as the free base. When the latter was further reduced with sodium in butyl alcohol 6-methyl ergoline (Formula II) resulted, owing to further reduction of the quinoline

* Reference (4) should have been designated Paper XV.

nucleus and the lactam group. Although this synthesis should result in a mixture of two racemic isomers since there are two centers of asymmetry in methyl ergoline, the properties of the substance obtained after recrystallization suggested homogeneity.



It gave color reactions which were very close to those given by lysergic acid itself. At this stage the attempt was made to obtain N-methyl ergoline by decarboxylation of dihydrolysergic acid but, as reported elsewhere (4), the latter was found not to decar-

boxylate on pyrolysis but to rearrange to a lactam with cleavage of Ring D. On the other hand, pyrolysis of lysergic acid itself has been found to result not only in loss of CO₂ but also of methylamine.

Parallel with the above studies a series of substances was synthesized leading to ergoline-7-carbonic acid as follows: *5,6-Benzoquinaldine-7-carbonic acid* (Formula III) was prepared by the Doebner reaction from 3-amino-1-naphthoic acid and acetaldehyde. It was further characterized as the *methyl* and *ethyl* esters. The 2-methyl group of this acid was smoothly oxidized to carboxyl by selenium dioxide (5) with formation of *5,6-benzoquinoline-2,7-dicarboxylic acid*. The nature of the latter was confirmed by its ready decarboxylation at the melting point with formation of the previously described monobasic *5,6-benzoquinoline-7-carbonic acid* (1).

On nitration of the dibasic acid a mixture of mononitro derivatives resulted which consisted essentially of *3'-nitro-5,6-benzoquinoline-2,7-dicarboxylic acid*. This was not purified as such, but its reduction product because of ready lactam formation was easily separated from other substances as the *lactam of 3'-amino-5,6-benzoquinoline-2,7-dicarboxylic acid* (Formula IV). The *methyl* and *ethyl* esters of the latter were also prepared. Hydrogenation of the lactam acid and its ester was then studied. When hydrogenation of the former was interrupted after absorption of 2 moles of H₂, an unstable 1,2,3,4-tetrahydro derivative resulted, which was isolated preferably as the hydrochloride. The *methyl* and *ethyl* esters proved to be more stable. The substance which was found to possess the most favorable properties of the series resulted from the complete hydrogenation of the ethyl ester. This product, since it proved to be the lactam of an amino group still of aromatic nature, must be *1,2,3,4,7,8,9,10-octahydro-3'-amino-5,6-benzoquinoline-2,7-dicarboxylic ethyl ester lactam* (Formula V).

Because of the unfavorable solubility relationships shown by the original, fully aromatic benzoquinoline acid and its esters, reduction with sodium and butyl alcohol proved to be impracticable. The reduction of the tetrahydro and octahydro derivatives was therefore studied. In these cases substances were obtained which gave the Keller reaction. The reaction most studied was

the reduction of the tetrahydro methyl ester. In this case the reaction mixture, which gave strongly the Keller reaction, yielded by careful manipulation a substance that approximated in composition that of the desired *ergoline-7-carbonic acid* (Formula VI). Unfortunately the yields obtained and the properties of the substance made very difficult its final purification. Since it has now become evident that the properties of lysergic acid fit best with those of an N-methyl ergoline-8-carbonic acid, this particular synthesis of 7-carbonic acids has not been pressed further.

It is of interest to mention at this point that all attempts to add methyl iodide to the benzoquinoline lactam acid (Formula IV) and its esters, as well as to their tetra and octahydro derivatives (Formula V), were unsuccessful. In most cases starting material was practically quantitatively recovered. This is possibly due to steric hindrance and is interesting in contrast with the behavior of lysergic acid, dihydrolysergic acid, their esters, and the dihydrolysergols which have been found to add methyl iodide readily. On the other hand, *2-methyl-5,6-benzoquinoline-7-carbonic ethyl ester* added methyl iodide readily to give the corresponding benzoquinaldinium methiodide derivative.

Simultaneously with the above, we have had in progress among other related subjects the synthesis of substances leading to ergoline-8-carbonic acid and ergoline-9-carbonic acid and their derivatives. This work will be presented at a later time.

EXPERIMENTAL

3'-Amino-5,6-Benzoquinolinium Methiodide-7-Carbonic Acid Lactam—3 gm. of the lactam prepared as previously described (1) were treated in a bomb tube with 15 cc. of methyl iodide at 100° for 18 hours. The bright orange-red crystalline precipitate was collected with ether. The yield was quantitative. Recrystallized from a large volume of water, the substance formed needles which melted at 291–292° with decomposition.

$C_{15}H_{11}ON_2I$.	Calculated.	C 49.72,	H 3.06,	N(CH ₃) 4.14
	Found.	" 49.98,	" 3.33,	" 2.12
				" 2.96

1-Methyl-2,3,4-Trihydro-3'-Amino-5,6-Benzoquinoline-7-Carbonic Acid Lactam—This substance was obtained by catalytic

hydrogenation of the methiodide. 0.285 gm. of the methiodide was suspended in 30 cc. of 0.5 per cent HCl and hydrogenated in the presence of 0.1 gm. of PtO_2 catalyst. The theoretical amount of hydrogen was absorbed in 3 to 4 hours. As the starting material dissolved, it was replaced by the hydrogenation product. The resulting suspension was heated at the end to dissolve the precipitate of colorless needles and the catalyst was filtered off. The clear solution was made slightly alkaline with ammonia and the precipitate of bright yellow crystals was collected. The yield was 90 per cent. The substance crystallizes from alcohol in plates which melted at $220\text{--}221^\circ$.

$\text{C}_{15}\text{H}_{14}\text{ON}_2$. Calculated. C 75.59, H 5.93, N(CH_3) 6.30
Found. " 75.48, " 5.61, " 4.94

6-Methyl Ergoline—A solution of 0.7 gm. of the previous N-methyl derivative in 100 cc. of dry *n*-butyl alcohol was treated with 5 gm. of sodium at the boiling point. After all the metal had dissolved, 100 cc. of water were added, and the butyl alcohol was removed *in vacuo*. The residual aqueous solution was extracted with benzene. The residue from the benzene extract was dissolved in a few cc. of ether. 2.5 cc. of water were added, followed by dilute HCl until the aqueous layer was just acid to Congo red. Beautifully crystalline 6-methyl ergoline hydrochloride separated on standing. The yield of crude salt was 75 mg. It was recrystallized several times from water with norit, and separated as glittering leaflets.

$\text{C}_{15}\text{H}_{18}\text{N}_2 \cdot \text{HCl}$. Calculated. C 68.54, H 7.29, N(CH_3) 5.71
Found. " 68.18, " 7.54, " 2.90

The free base was obtained by addition of alkali to an aqueous solution of the salt. It separated as a flocculent white precipitate. Recrystallized from methyl alcohol or chloroform, it melted at $210\text{--}212^\circ$. The pure substance is practically insoluble in ether, whereas the crude mixture of products obtained by the sodium-butyl alcohol reduction is readily soluble. The substance, although a racemic mixture, appeared to be otherwise homogeneous.

$\text{C}_{15}\text{H}_{18}\text{N}_2$. Calculated. C 79.59, H 8.02, N(CH_3) 6.64
Found. " 79.54, " 7.57, " 5.97

6-Methyl ergoline gives the Keller test but there is a suggestion that the color shade in the acetic acid layer is a little more in the purple than that given by lysergic acid. It gave a definite melting point depression with ergoline itself. It may be mentioned at this point that *ergoline* more recently purified by repeated recrystallization of its hydrochloride has since been found to melt at 201–203° instead of at 175–183° as previously given (1).

5,6-Benzoquinaldine-7-Carbonic Acid—This acid was prepared by the Doebner reaction. 30 gm. of the finely powdered sulfate of 3-amino-1-naphthoic acid were mixed with 30 gm. of paraldehyde and 60 cc. of HCl (sp. gr. 1.19) and the mixture was heated under a reflux at 110–120°, with mechanical stirring, for 6 to 8 hours. The reaction mixture was diluted with 500 cc. of water, brought to a boil, and then filtered. The filtrate was neutralized to Congo red with NaOH solution and, after cooling, the precipitate was filtered. The crude product was recrystallized several times from dilute HCl as the hydrochloride. The latter was finally decolorized with norit in dilute HCl solution and then neutralized with NaOH as before to give the free quinaldine acid. The yield was about 9 gm.

For analysis it was recrystallized from a small volume of pyridine and separated as transparent rods which melted at 313–315° with decomposition.

$C_{16}H_{11}O_2N$. Calculated, C 75.92, H 4.68; found, C 76.15, H 4.65

The hydrochloride formed needles from dilute HCl, which melted at 314–316° with decomposition.

$C_{16}H_{11}O_2N \cdot HCl$. Calculated, C 65.80, H 4.42; found, C 65.77, H 4.75

The Methyl Ester—The finely powdered hydrochloride of the acid was suspended in a large volume of absolute methyl alcohol and refluxed with a continuous stream of HCl until a clear solution resulted. The resulting HCl salt was converted as usual into the free quinaldine ester. Recrystallized from alcohol, it formed needles melting at 114–116°.

$C_{16}H_{13}O_2N$. Calculated, C 76.46, H 5.22; found, C 76.44, H 4.96

The Ethyl Ester—The ethyl ester prepared in the same manner after recrystallization from alcohol melted at 103–104°.

$C_{17}H_{15}O_2N$. Calculated, C 76.94, H 5.70; found, C 77.32, H 6.07

5,6-Benzoquinaldinium Methiodide-7-Carbonic Ethyl Ester—The ethyl ester when heated at 100° with an excess of methyl iodide gave the methiodide in good yield. Recrystallized from water, it melted at 201–203° with decomposition.

$C_{18}H_{13}O_2NI$. Calculated, C 53.06, H 4.46; found, C 52.5, H 4.45

5,6-Benzoquinoline-2,7-Dicarboxic Acid—The 2-methyl group in the benzoquinaldine acid was readily oxidized to carboxyl by selenium dioxide, either as the ester in amyl alcohol or xylene solution, or better as the free acid in pyridine solution. Ordinary so called c.p. pyridine contains homologous methyl pyridines which were oxidized to the corresponding pyridine acids (5) at the same time, but this did not affect the main reaction and the products could be easily separated.

20 gm. of benzoquinaldine-7-carboxic acid were dissolved in 150 cc. of boiling pyridine and cooled. 40 gm. of selenium dioxide dissolved in 20 cc. of warm water were added in small portions and the mixture was then refluxed for 8 hours. The precipitated selenium was collected and found to weigh 11.5 gm.; calculated, 10 gm. The clear filtrate was concentrated *in vacuo*. The residue formed a crystalline cake which was dissolved in water. Dilute HCl was added until slightly acid to Congo red, and the precipitate was filtered off. The crude dibasic acid was washed with water and dried *in vacuo* at 100°. The yield was practically theoretical. It melted at 258° with evolution of CO_2 , resolidifying as the monobasic 7-carboxic acid which melted at about 300° as previously described (1).

$C_{18}H_9O_4N$. Calculated, C 67.40, H 3.40; found, C 67.66, H 3.19

The substance formed a beautifully crystalline monosodium salt from an acetic acid-sodium acetate buffer.

3'-Nitro-5,6-Benzoquinoline-2,7-Dicarboxic Acid—The nitration was performed as described for the monocarboxic acid, with fuming nitric acid (sp. gr. 1.58) at 0°, and the resulting solution was poured into ice water. It was purified by reprecipitation from alkaline solution with dilute HCl.

$C_{18}H_7O_6N_2$. Calculated, C 57.68, H 2.58; found, C 57.74, H 3.06

The substance was presumably not homogeneous.

S'-Amino-5,6-Benzoquinoline-2,7-Dicarboxylic Acid Lactam—10 gm. of the above nitro derivative were dissolved in a mixture of 50 cc. of water and 15 cc. of concentrated ammonium hydroxide. The solution was added slowly with shaking to a suspension of ferrous hydroxide prepared from a mixture of 100 gm. of ferrous sulfate in 250 cc. of water and 100 cc. of ammonium hydroxide. The mixture was heated to about 70° and treated with 65 cc. of 50 per cent NaOH. It was then boiled for 5 minutes and filtered. The clear, light red filtrate was acidified to Congo red with HCl which produced a flocculent red precipitate gradually turning to a tan color. After the mixture was boiled for 5 minutes to complete lactamization and cooled, the crude product was collected with water. The yield was 7.8 gm., which represented an over-all yield from benzoquinoline-7-carboxylic acid of about 70 per cent. The substance was soluble only in concentrated H₂SO₄ and dilute aqueous pyridine or piperidine solutions. It was purified through the sparingly soluble ammonium salt, and by precipitation from solution in dilute pyridine with HCl.

The ammonium salt melted with decomposition at 273–276°.

C ₁₆ H ₁₁ O ₃ N ₃ .	Calculated.	C 64.03, H 3.94, N 14.95
	Found.	" 63.9, " 3.51, " 14.93

The free acid, which was yellow in color, was dried at 100° *in vacuo* and melted at 270–271° with evolution of CO₂ and then resolidified. The resulting benzoquinoline lactam melted at 280–281° as previously described (1).

C ₁₆ H ₉ O ₃ N ₂ .	Calculated, C 68.16, H 3.05; found, C 67.9, H 3.17
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The Methyl Ester—The free acid was esterified by passing HCl through the boiling methyl alcoholic suspension or by refluxing in methyl alcohol containing 6 per cent of H₂SO₄. The resulting sparingly soluble methyl ester salts were hydrolyzed when treated with water. The bright yellow ester was recrystallized from pyridine and melted at 305–307°.

C ₁₈ H ₁₀ O ₃ N ₂ .	Calculated, C 69.04, H 3.62; found, C 68.30, H 3.62
	" " 68.23, " 3.52

The Ethyl Ester—This ester was prepared in the same way. Recrystallized from pyridine or glacial acetic acid, it melted at 275–277°.

$C_{17}H_{15}O_3N_2$. Calculated, C 69.84, H 4.14; found, C 69.24, H 4.18
" " 69.56, " 4.58

1,2,3,4-Tetrahydro-3'-Amino-5,6-Benzoquinoline-2,7-Dicarboxic Acid Lactam—1.5 gm. of the previous benzoquinoline lactam acid suspended in about 50 cc. of acetic acid were hydrogenated at 60° with 0.5 gm. of PtO_2 catalyst. The solution absorbed 460 cc. of H_2 in 1 hour, and the absorption which had slowed down was interrupted at this point. Calculated for 2 moles of H_2 , 413 cc. The clear solution was filtered from the catalyst and enough HCl was added to cause complete precipitation of the acid salt. The yield of hydrochloride of the tetrahydro compound was 1.3 gm.

The free tetrahydro acid was found to be very sensitive to air, as in the case of tetrahydroquinaldinic acid (6). It was obtained by treating an aqueous suspension of the hydrochloride with sodium acetate solution. The substance melted at 237–239°, and was lemon-yellow in color. However, it has been very difficult to obtain satisfactory analytical figures with the acid itself. Better results were obtained with the esters.

The Methyl Ester—This ester was obtained with methyl alcohol and HCl, and also by catalytic reduction of the parent benzoquinaldinic acid ester. Recrystallized from methyl alcohol, the yellow tetrahydro ester melted at 234–236°.

$C_{16}H_{14}O_3N_2$. Calculated, C 68.05, H 5.00; found, C 68.38, H 5.06

The Ethyl Ester—Both methods of preparation were also used in this case. Recrystallized from ethyl alcohol or pyridine, the substance melted at 240–242°.

$C_{17}H_{16}O_3N_2$. Calculated, C 68.88, H 5.44; found, C 69.43, H 5.38
" " 68.46, " 5.15

1,2,3,4,7,8,9,10-Octahydro-3'-Amino-5,6-Benzoquinoline-2,7-Dicarboxic Acid Lactam Ethyl Ester—When the above catalytic hydrogenation in the case of the ethyl ester was continued for a long time (30 to 40 hours), a crystalline substance was isolated in about 30 per cent yield. Analysis showed it to be an octahydro derivative. Over this long period the apparent absorption of H_2 corresponded to about 5 moles, possibly due to leakage. It formed long, pale yellow needles from ethyl alcohol, which melted at 232–236°.

$C_{17}H_{21}O_2N_2$. Calculated, C 67.96, H 6.72; found, C 68.29, H 6.80

This substance, after cleavage with alkali, gave the usual reactions of an aromatic amino derivative. On reduction with sodium and alcohol the formation of an indole derivative also demonstrates the aromatic character of the amino group.

Ergoline-7-Carbonic Acid—0.7 gm. of 1,2,3,4-tetrahydro-3'-amino-5,6-benzoquinoline-2,7-dicarboxylic acid lactam methyl ester was dissolved in 100 cc. of dry *n*-butyl alcohol and brought to a boil. 5 gm. of sodium were added and the mixture was shaken vigorously. The color changed to an orange-red at first and finally a clear, almost colorless solution resulted. After addition of water and removal of butyl alcohol *in vacuo*, the residual aqueous alkaline solution was extracted successively with benzene and ether to remove by-products and then filtered. The solution was then made just acid to Congo red with H_2SO_4 , which caused precipitation of dark colored, amorphous material that was filtered off. Sodium carbonate solution was added to the filtrate until just alkaline to phenolphthalein. On concentration a residue was obtained which was dried and ground. This material was extracted three times with 25 cc. of boiling alcohol, and the combined extracts were concentrated *in vacuo*. The red, resinous residue was dissolved in a few cc. of water. Dilute HCl was cautiously added until just acid to Congo red and after standing at 0° the solution was filtered from a dark colored, amorphous precipitate. The filtrate was concentrated *in vacuo* to a thick syrup, and a few drops of HCl (1:1) were added. After standing at 0° for several days, crystalline material separated, which was collected. The yield was only 10 mg. After recrystallization from dilute HCl it formed glittering leaflets. Its color reactions were practically identical with those given by lysergic acid.

$C_{15}H_{16}O_2N_2 \cdot HCl$. Calculated, C 61.52, H 5.86; found, C 60.9, H 5.14

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THE ENZYMATIC SYNTHESIS AND HYDROLYSIS OF CHOLESTEROL ESTERS IN BLOOD SERUM*

By WARREN M. SPERRY AND V. A. STOYANOFF

(From the Chemical Laboratory, Babies Hospital, and the Department of
Biochemistry, College of Physicians and Surgeons, Columbia University,
New York)

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In recent publications (1, 2) we have shown that bile salts exert a marked influence on the proportion between the cholesterol fractions in incubated human and dog sera. In small concentrations all of the bile salts studied inhibit, increasingly with increasing amounts, the esterification of free cholesterol which occurs normally during incubation of both types of serum. In larger concentrations some of them promote hydrolysis of cholesterol esters in dog serum but not in human serum. It is probable that these reactions are involved, directly or indirectly, in the metabolism of cholesterol *in vivo*. For example, if esterification of free cholesterol goes on continuously *in vivo* as it does *in vitro*, cholesterol esters must be removed continuously from the serum in order to maintain the proportion between combined and free cholesterol within the narrow limits observed in healthy human beings (3). In hepatic disease bile salts, accumulating in the serum, may inhibit the esterification as they do *in vitro* without affecting the mechanism for removing cholesterol esters. The result would be a decrease in the proportionate amount of combined cholesterol such as is actually found. On the basis of such considerations it seemed worth while to study the reactions in more detail and in the present communication we describe a series of experiments designed to shed further light on the mechanisms involved.

1. The spontaneous esterification of free cholesterol on in-

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cubation of serum and the absence of any reaction in serum which has been heated at 60° for 1 hour are strong evidence for the enzymatic nature of the reaction (4). In this investigation mixtures of human or dog serum with heat-inactivated serum of either the same or the opposite species were incubated. Both human and dog sera promoted esterification of free cholesterol in heat-inactivated serum of each species. The finding provides further proof of the existence in serum of an enzyme or enzyme system which may be called the "esterifying factor."

2. The ability of larger concentrations of taurocholate and glycocholate to promote hydrolysis of cholesterol esters in dog serum but not in human serum can be explained by the hypothesis that a different enzyme or enzyme system—a "hydrolyzing factor"—is also present in dog serum but absent from human serum. Evidence in support of this theory was obtained in the following experiments: (a) Mixtures of human and dog sera were incubated in the presence of taurocholate. Complete or nearly complete¹ hydrolysis of the cholesterol esters of both the human and admixed dog sera resulted. (b) Mixtures of dog serum with heat-inactivated human or dog serum were incubated in the presence of taurocholate. There was complete hydrolysis of all of the cholesterol esters of such mixtures. (c) Dried dog serum was added in small amount to heat-inactivated dog serum which was then incubated in the presence of taurocholate. Marked (usually complete) hydrolysis of cholesterol esters resulted. (d) Globulin fractions from dog serum in the presence of taurocholate catalyzed complete hydrolysis of cholesterol esters in human

¹ In previous publications we have referred frequently to the hydrolysis of cholesterol esters in dog serum in the presence of larger amounts of glycocholate and taurocholate as "complete." Actually the ratio of combined to free cholesterol usually varies from 0 to 0.06. We thought for a time that the small amount of unhydrolyzed ester, which such ratios indicate, represented experimental error; but, if that were true, an equal variation in the opposite direction should have been and has not been observed. In few experiments did the apparent free cholesterol concentration exceed the total cholesterol concentration. Unless in our analytical procedure there is a systematic loss of free cholesterol, for which there is no evidence (5), the hydrolysis is usually not quite complete. For convenience in discussion, however, we shall continue to use the term "complete hydrolysis" in referring to experiments yielding ratios within the range indicated.

serum and in heat-inactivated human or dog serum. Fractions prepared in exactly the same way from human serum were inert.

3. The dried dog serum and the globulin fractions from human and dog sera were incubated with unheated and heat-inactivated sera without taurocholate. All of the preparations inhibited, sometimes almost completely, the esterification of free cholesterol in unheated serum. We have no explanation for this unexpected result. The fact that the human globulin, which contained no "hydrolyzing factor," showed the inhibitory effect just as did the dog globulin demonstrates that the "hydrolyzing factor" is not responsible. None of the fractions catalyzed esterification in heat-inactivated serum. The finding presents strong evidence for the non-identity of the "hydrolyzing" and "esterifying" factors, since the dog preparations were rich in the former.

4. Serum was incubated alone until esterification had approached a maximum, taurocholate was added, and incubation was continued for an additional period. With human serum taurocholate had little effect. With dog serum the results varied widely from experiment to experiment and from replicate to replicate in the same experiment. Usually (but not always) there was some hydrolysis, but only once were cholesterol esters split completely as they would have been uniformly if taurocholate had been added at the beginning of incubation. The result supports the view that two factors are present in dog serum and indicates that the one concerned with the splitting of cholesterol is inactivated during the preliminary incubation period to an extent which varies with conditions not yet understood. If so, the addition of the active factor along with taurocholate should promote complete hydrolysis. To test the point dog globulin was added along with taurocholate at the end of the preliminary period of incubation in some of the experiments. Without exception complete hydrolysis of cholesterol esters occurred.

5. The rates of esterification of free cholesterol and of hydrolysis of cholesterol esters in the presence of bile salt were studied. The esterification is relatively slow, requiring 3 to 4 days to approach a maximum. The hydrolytic reaction is by contrast very rapid, being half complete within 5 minutes and approaching a maximum in 4 to 8 hours.

All of the findings are in accord with the hypothesis that the

esterification of free cholesterol and the hydrolysis of cholesterol esters in dog serum in the presence of bile salt are in effect independent reactions catalyzed by different enzymes or enzyme systems, both of which are present in dog serum and of which only the one concerned with esterification is present in human serum. Speculation as to the mechanisms of these enzymatic reactions, attractive as it is, must be deferred until it becomes warranted by further evidence.

EXPERIMENTAL

As the work has involved several thousand cholesterol determinations, it will be impossible to present the data in detail. Fortunately in most instances experiments repeated under the same conditions gave essentially the same result; hence the findings may be described by illustrative examples.

Serum was incubated at 37–38° alone or with the addition of various substances for 1 to 3 days in tightly stoppered test-tubes. The cholesterol fractions were determined by the method of Schoenheimer and Sperry (6) in control samples before, and in the experimental samples after incubation. Most of the experiments were performed in duplicate; in a few instances where greater precision was desired three or four replicates were incubated together. Control analyses were carried out in triplicate.

The first part of the work was not performed under sterile conditions, and to reduce the chance of bacterial growth the time of incubation was restricted to 1 day. As in the preceding investigation (2) which was carried out concurrently with many of the experiments of the present study, occasional samples became cloudy. This degree of infection appeared to have no influence on the findings, since the agreement between duplicates when only one of them had clouded was satisfactory. Furthermore after the discovery that sodium ethyl mercurithiosalicylate (added in the proportion of 0.1 cc. of 1 per cent solution per 10 cc. of serum) protected against infection and had little, if any, influence on the esterification, most of the experiments were repeated in the presence of this preservative and with a 2 or 3 day incubation period. The results were unchanged.

In some of the experiments in which the samples were incubated 1 day the amount of esterification was small. The finding was

explained later by studies of the rate of esterification, which proceeds slowly and approaches completion only in 3 days. This circumstance does not invalidate the 1 day experiments, because the period of incubation was the same for all samples in each experiment and the effects of various substances were qualitatively the same for 1 day and 3 day incubation periods. Furthermore much of the work was concerned with the splitting of cholesterol esters in the presence of bile salt, a reaction which proceeds much more rapidly than the esterification and is virtually complete within 4 hours.

With the exception of a few early experiments in which glycocholate was used, taurocholate was the only bile salt employed in this investigation. It was selected because of those studied it appears to have the most marked effect on the hydrolysis of cholesterol esters in dog serum (2). 0.075 cc. of a 0.065 M solution of bile salt was added to 0.3 cc. of serum. The final concentration was therefore 0.013 M except in those experiments in which other solutions were also added; in these the concentration (minimum 0.011 M) was well within the range which promotes complete splitting of cholesterol esters in dog serum (see Fig. 3 (2)).

Serum which had been heated at 55–60° for 1 hour was employed in many of the experiments. Many samples of such heat-treated human and dog sera have been incubated alone and with the addition of bile salts. Without exception no esterification occurred in the absence of bile salts and there was no hydrolysis of cholesterol esters in dog serum in the presence of bile salts. It seems certain therefore that the active factors are inactivated by the heating process, and such heat-inactivated serum presents an ideal medium for testing the activity of various preparations.

1. Esterification of Free Cholesterol in Heat-Inactivated Serum Promoted by Unheated Human or Dog Serum—Mixtures of human or dog serum² with heat-inactivated serum of either the same or

² All of the human serum used in this investigation was from venous blood, while most samples of dog blood were taken from the femoral artery. The remote possibility that the apparent differences between human and dog sera might actually be differences between venous and arterial sera was ruled out by direct comparisons of venous and arterial sera taken simultaneously from the same dog. The cholesterol concentration was the same, esterification proceeded to the same degree in the absence of bile salt, and there was complete hydrolysis of cholesterol esters in the presence of taurocholate in both types of serum.

the opposite species were incubated. Thirty-three experiments, about equally distributed among the four different mixtures, were carried out. The proportion was varied from 3:2 to 1:6 parts of unheated to heat-inactivated serum, respectively. In twenty experiments it was 1:1. A representative experiment is given in Table I.

In this and all other experiments of the investigation the degree of esterification (or hydrolysis) was calculated from free (or combined) cholesterol values corrected for deviations in the total cholesterol concentration from the control value for reasons which

TABLE I

Esterification of Free Cholesterol in Heat-Inactivated Human Serum Promoted by Unheated Human Serum

	Cholesterol			Esteri- fication per cent
	Total	Free	Corrected free	
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
Control.....	199.9	53.1		
Unheated serum incubated 3 days.....	210.1	26.5	25.2	52.5
Mixture (1:1) of unheated and in- activated serum incubated 3 days.....	204.4	32.0	31.3	41.1
Theoretical values if only un- heated portion had reacted.....			39.2	26.3
Apparent esterification in inacti- vated portion.....			7.9	29.7

are omitted in order to save space. The corrections were usually small. In interpreting the data it was assumed that esterification of the free cholesterol of the unheated portion of the mixture took place at the same rate as in serum incubated alone. The assumption seems justified, since there is no reason to anticipate a higher rate of esterification, while a lower rate would reduce the apparent esterification of free cholesterol supplied by the heat-inactivated serum.

No differences among the four types of mixtures could be made out. In four of the experiments there was no apparent esterifica-

tion of free cholesterol supplied by the inactive component of the mixture and in five the value was less than 10 per cent and of questionable significance. These nine experiments involved only 1 day of incubation and, as the degree of esterification in control samples was small, the error in calculating the apparent esterification in the inactivated part of the mixture was high. Without exception in experiments carried out with 3 days incubation there was a large apparent esterification of the "inactivated" free cholesterol with a maximum of 64.7 per cent. The average of fourteen such experiments was 33.1 per cent, while the average of the entire series was 20.3 per cent.

2, a. *Hydrolysis of Cholesterol Esters in Human Serum Promoted by Dog Serum in Presence of Taurocholate*—Nine samples of

TABLE II

Hydrolysis of Cholesterol Esters in Human Serum Promoted by Dog Serum in Presence of Taurocholate

Treatment	Cholesterol			Ratio, combined to free
	Total	Free	Combined	
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
Control.....	194.8	56.3	138.5	2.46
Incubated with taurocholate.....	146.8	42.3	104.5	2.47
" " " and				
dog serum.....	143.5	141.4	2.1	0.01

human serum were mixed with dog serum in equal proportions and incubated with bile salt. The result of a representative experiment is given in Table II. The ratio of combined to free cholesterol varied from 0 to 0.06 with an average of 0.02, showing complete or nearly complete¹ splitting of the cholesterol esters of the human as well as of the admixed dog serum. The ratio did not change significantly from the control value in human serum incubated with bile salt alone, in accord with previous findings (see Fig. 3 (2)) and many other such experiments carried out during the course of this investigation.

2, b. *Hydrolysis of Cholesterol Esters in Heat-Inactivated Human and Dog Sera Promoted by Dog Serum in Presence of Taurocholate*—In six experiments mixtures of dog serum and heat-inacti-

vated human serum in the proportion of 1:1 or 1:2 were incubated with taurocholate. The ratio of combined to free cholesterol varied from 0 to 0.05. In control experiments with the situation reversed (mixtures of human serum and heat-inactivated dog serum) the ratio did not change appreciably from the original level during incubation. In five similar experiments with mixtures of unheated and heat-inactivated dog sera the ratio varied from 0 to 0.06.

2, c. Effect of Dried Dog Serum on Hydrolysis of Cholesterol Esters—Six samples of dog serum were frozen and taken to dryness in *vacuo*. The dry powder (5 mg.) was dissolved in unheated and heat-inactivated portions (0.3 cc.) of the same serum which had been kept cold during the day or two required for drying. Taurocholate was added and the samples were incubated. Cholesterol esters were hydrolyzed, completely in four experiments and to a marked degree in two. The ratios of combined to free cholesterol before incubation were 2.72, 2.60, 2.69, 2.88, 2.16, and 2.66; after incubation with taurocholate and dried serum they were respectively 0, 0.01, 0.02, 0.07, 1.09, and 1.39. When the dried serum was dissolved in water in approximately the proportion of the original serum and incubated with taurocholate, marked hydrolysis of cholesterol esters occurred.

Portions of the dried serum were extracted exhaustively with acetone and ether. The resulting residues behaved like the unextracted material and, furthermore, the analytical data indicated that cholesterol was still present. On repeating the extraction quantitatively it was found that little, if any, of the serum lipids is removed at room temperature by acetone, ether, or ethylene chloride. However, if the dried serum is first dissolved in water, the lipids may be extracted by treating with an excess of acetone, centrifuging, and washing the precipitate with acetone and ether. Two such lipid-free protein preparations were studied in the same manner as was the whole dried serum. They appeared to be inert, promoting no hydrolysis in inactivated serum. Several analogous preparations from untreated serum behaved in the same manner.

2, d. Effect of Serum Globulin on Hydrolysis of Cholesterol Esters—Serum was treated with an equal volume of saturated ammonium sulfate; the precipitate was centrifuged down at high

speed, dissolved in, and dialyzed against 0.9 per cent NaCl under reduced pressure (7) until the dialysate was almost sulfate-free. The globulin solution was frozen and taken to dryness *in vacuo*. The residue was powdered and employed in studies like those carried out with dried whole serum, 5 mg. being dissolved in 0.3 cc. of serum. Five such preparations from dog serum and an equal number from human serum were studied. Each was incubated with unheated and heat-inactivated human and dog sera, with the addition of taurocholate.

The result of a typical experiment is shown in Table III. It is evident that dog globulin catalyzed the hydrolysis of cholesterol

TABLE III
Effect of Serum Globulin Fractions on Hydrolysis of Cholesterol Esters in Serum

Treatment of serum	Substances added	Ratio,* combined cholesterol to free cholesterol	
		Human serum	Dog serum
1. None	None (control)†	2.61	2.41
2. "	" (incubated)	4.94	10.81
3. "	Taurocholate	2.69	0.01
4. "	" and dog globulin	0.05	0.04
5. "	" " human "	2.59	0.04
6. Heated	" " dog "	0.00	0.03
7. "	" " human "	2.61	2.43

* An increased ratio indicates esterification of free cholesterol; a decreased ratio indicates hydrolysis of cholesterol esters.

† All samples except the control were incubated 3 days.

esters in human serum (compare Lines 3 and 4) and in heat-inactivated human and dog serum (Line 6). Human globulin had no effect in catalyzing hydrolysis (Lines 5 and 7) though it did not interfere with the normal splitting of esters in dog serum in the presence of taurocholate (Line 5).

3. The dried dog serum and the globulin fractions were incubated with unheated and heat-inactivated serum without taurocholate. The effect of the globulin fractions is shown by the result of a typical experiment (Table IV). Both human and dog globulins inhibited esterification of free cholesterol in each type of serum (compare Lines 3 and 4 with Line 2). In some experi-

ments the inhibition was almost complete. Neither type of globulin promoted esterification in heat-inactivated serum (Lines 5 and 6). Dried dog serum also inhibited esterification, usually almost completely. For example, in one experiment the control ratio of combined to free cholesterol was 2.69, the ratio after incubation of the serum alone was 4.06, while with the addition of dried serum it was 2.85. The dried serum showed no ability to catalyze esterification in heat-inactivated serum.

All of the globulin fractions hydrolyzed tributyrin (8, 9), those from human serum apparently possessing greater activity than those from the dog. The factor concerned with splitting chole-

TABLE IV

*Effect of Serum Globulin Fractions on Esterification of Free Cholesterol in Serum**

Treatment of serum	Substances added	Ratio, combined cholesterol to free cholesterol	
		Human serum	Dog serum
1. None	None (control)†	2.61	2.41
2. "	" (incubated)	4.94	10.81
3. "	Human globulin	3.69	5.75
4. "	Dog "	3.96	5.37
5. Heated	Human "	2.59	2.35
6. "	Dog "	2.54	2.37

* This experiment was carried out simultaneously with the one described in Table III on the same sera and globulin fractions.

† All samples except the control were incubated 3 days.

terol esters cannot be tributyrinase, since the former was not present in human globulin. Moreover the factor responsible for esterification of free cholesterol is not the enzyme which hydrolyzes tributyrin, since the former was absent from all of the globulin preparations.

4. *Effect of Taurocholate Added to Serum after Esterification Has Taken Place*—Samples of dog serum were incubated for 2 or 3 days, taurocholate was added, and incubation was continued for 2 days more. Control samples without bile salts were analyzed at the end of the preliminary and total incubation periods. The experiments were carried out in triplicate, quadruplicate, or, in one experiment, sextuplicate.

The findings in eight such experiments, in contrast with most of those obtained in this investigation, are characterized by a large variation among the average values of different experiments and among the values obtained in replicates in the same experiment. There was usually some hydrolysis, but cholesterol esters were split completely in only one experiment. In some experiments four of the dog globulin preparations described above were added along with taurocholate at the end of the preliminary period (5 mg. to

TABLE V
Rate of Synthesis and Hydrolysis of Cholesterol Esters in Dog Serum

Time of incubation	Esterification (without bile salt)	Hydrolysis (with taurocholate)
	<i>per cent</i>	<i>per cent</i>
0 min.*		3.7
5 "		56.3
10 "		68.9
15 "		70.3
20 "		73.4
30 "		75.6
1 hr.		92.5
2 hrs.	7.4	
4 "	12.2	98.3
6 "	15.2	
8 "	18.9	96.4
1 day	47.2	98.7
2 days	57.8	99.2
3 "	67.4	
6 "	69.1	
7 "	66.4	
8 "	68.0	

* Analyzed immediately after mixing the serum and bile salt solution.

0.3 cc. of serum). The samples were incubated for an additional period along with those containing only taurocholate. Without exception complete hydrolysis of cholesterol esters occurred.³

³ The globulin fractions were nearly a year old when these experiments were carried out and it was necessary to ascertain whether they were still active. They had been kept at room temperature in stoppered tubes. Samples of heat-inactivated serum to which the globulin preparations and taurocholate had been added were incubated as controls in each of these experiments. Cholesterol esters were hydrolyzed completely. The result demonstrates the stability of the "hydrolyzing factor."

The effect of taurocholate added to human serum after a preliminary period of incubation was studied in five experiments. Triplicate and quadruplicate determinations agreed satisfactorily and the findings were essentially the same throughout. The average esterification in the preliminary period was 41.0 per cent, in the total period (without bile salt) 44.8 per cent, and with taurocholate 40.0 per cent.

*5. Rate of Synthesis and Hydrolysis of Cholesterol Esters—*Samples of serum with and without added taurocholate were analyzed after varying intervals in the incubator. The result of a typical experiment is shown in Table V. The values for esterification and hydrolysis are the percentage decreases in the respective free and combined cholesterol concentrations from the control amounts.

The esterification of free cholesterol is a relatively slow reaction, approaching a maximum in 3 days. All of the experiments (one on human and five on dog serum) gave curves of the same general shape, though there was considerable variation in the maximum degree of esterification. The hydrolysis on the other hand is very rapid; it was consistently over 50 per cent complete in 5 minutes and reached a maximum in 4 to 8 hours.

SUMMARY

Changes in the proportion between the cholesterol fractions occurring in human and dog blood sera on incubation at 37–38° were studied with and without the addition of various substances.

No esterification of free cholesterol or hydrolysis of cholesterol esters (in dog serum with added bile salt) occurs on incubation of serum which has been heated at 55–60° for 1 hour, and it serves, therefore, as an excellent medium for studying the activity of various preparations.

Both human and dog sera promote esterification of free cholesterol in heat-inactivated serum of each species.

Dog serum in the presence of taurocholate promotes hydrolysis of cholesterol esters in human serum and in heat-inactivated human and dog sera.

In the presence of taurocholate small amounts of dog serum, dried *in vacuo* at low temperature, catalyze hydrolysis of cholesterol esters in inactivated portions of the same serum.

Globulin fractions from dog serum in the presence of taurocholate catalyze hydrolysis of cholesterol esters in human serum and in heat-inactivated human and dog sera. Globulin fractions from human serum prepared in exactly the same way do not promote hydrolysis.

Dried dog serum and globulin fractions from human and dog sera inhibit the spontaneous esterification of free cholesterol in unheated serum and do not catalyze esterification in heat-inactivated serum.

Hydrolysis of cholesterol esters in dog serum in the presence of bile salt is a rapid process, being half completed in 5 minutes and reaching a maximum within 8 hours or less. The esterification of free cholesterol is relatively slow, approaching a maximum in 3 days.

All of the findings may be correlated with the hypothesis that the esterification of free cholesterol and the hydrolysis of cholesterol esters are in effect independent reactions catalyzed by different enzymes or enzyme systems, both of which are present in dog serum and of which only the one concerned with esterification is present in human serum. The factor in dog serum concerned with the hydrolysis of cholesterol esters is inactivated to a variable degree by incubation of serum for 2 to 3 days.

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THE PURIFICATION OF THROMBIN*

BY WALTER H. SEEGER, K. M. BRINKHOUS, H. P. SMITH, AND
E. D. WARNER

(From the Department of Pathology, State University of Iowa, Iowa City)

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In a recent article we described a simple procedure for the purification of prothrombin (3). The product obtained was relatively stable and was water-soluble. On addition of thromboplastin and calcium, a powerful solution of thrombin formed. It is our experience, however, that on addition of calcium alone the conversion of prothrombin into thrombin is highly incomplete.

The thrombin solution obtained on mixing prothrombin, calcium, and thromboplastin is centrifuged and then precipitated and dried by the addition of acetone. Thrombin, being water-soluble, can then be extracted from the precipitate, leaving behind a number of impurities which are rendered insoluble by the acetone. Additional impurities can be removed by addition of dilute acetic acid to the extract. The thrombin is then reprecipitated with acetone and dried.

EXPERIMENTAL

In our earlier experiments we used cephalin as a source of thromboplastic activity. This substance has the advantage over simple organ extracts in that its composition is understood, and it can be prepared in a reasonable state of purity. However, we found that with cephalin the conversion of prothrombin into thrombin is usually incomplete, and always requires many hours during which much of the thrombin formed disintegrates. Crude organ extracts, on the other hand, contain thromboplastin in a rapidly acting form, but they also contain substances which

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destroy thrombin. We have attempted to separate the thromboplastic material from the antithrombic substances. The thromboplastin of lung can be precipitated with acids (Mills (2)). Using a saline extract of fresh ox lung, we have separated the thromboplastic material by precipitation with dilute acetic acid. The product is largely free of antithrombin. Admittedly, it does not react as promptly as before precipitation, yet its action is more rapid and complete than that of cephalin.

Preparation of Thromboplastin—As a preliminary step, a crude extract of beef lung is prepared as previously described (4). To 100 gm. of fresh ground beef lung add 100 cc. of NaCl solution (0.9 per cent). Place in the ice box and stir at intervals during the next 24 hours. Centrifuge. Dilute 10 cc. of the clear supernatant fluid with 90 cc. of water. Add 2 cc. of 1 per cent acetic acid. The precipitate is washed twice in 40 cc. of distilled water. The pH of the last wash water is about 6.5 to 7.0, though we have made no effort to control this. If the precipitate is then extracted with saline at pH 7.3, active thromboplastin dissolves, and this can be used for the conversion of the purified prothrombin into thrombin. However, to avoid excessive dilution, we make it a practice to add the moist precipitate to the prothrombin-calcium solution. Undissolved material is removed by the centrifuge after the prothrombin conversion is completed.

Conversion of Prothrombin into Thrombin—Prothrombin is purified as previously described (3), though on a somewhat larger scale. To 1.5 liters of oxalated beef plasma add 15 liters of cold distilled water and 1 per cent acetic acid until the pH is 5.3. The precipitate from the cold mixture settles within 2 to 3 hours. It is removed and dissolved in 400 to 500 cc. of oxalated saline. Insoluble protein is discarded. The solution is mixed with 100 to 125 cc. of $Mg(OH)_2$ suspension. The adsorbed prothrombin is liberated in water by the aid of CO_2 . When this crude prothrombin solution is dialyzed at 5° for 24 to 48 hours against repeated changes of distilled water, a considerable amount of inert material precipitates out and is discarded. Further purification is effected by the addition of 1 per cent acetic acid in the cold until the pH is brought to 5.3. Most of the prothrombin precipitates, and is then collected and dissolved in 30 to 40 cc. of 0.9 per cent NaCl and 0.15 per cent $Ca(NO_3)_2$. The pH is

adjusted to 7.0 to 7.2 with the aid of 0.1 N NaOH and phenol red. To the solution is now added the thromboplastin precipitate prepared as above from approximately 10 gm. of fresh beef lung.

The rate at which prothrombin is converted into thrombin depends upon factors not yet wholly understood. With suitable mixtures of plasma and crude lung extract, conversion is complete in 1 to 2 minutes. Our purified prothrombin appears to be somewhat less reactive, for, when mixed with crude lung extract and calcium, a period of 5 to 10 minutes must be allowed to effect complete conversion. With thromboplastin purified as above, the conversion is still slower. 50 per cent is converted in about 20 minutes, and 2 hours are needed for complete conversion. There is no loss of thrombin if an extra hour or two are allowed. The slow conversion is no doubt due in part to the relative insolubility of the purified thromboplastin. The conversion, though slow, is nevertheless more complete than with cephalin, and the reagents are almost entirely free of antithrombin, an obvious advantage over using crude saline organ extracts.

Purification of Thrombin—The above thrombin solution is chilled and treated with 3 volumes of cold acetone. The precipitate, which is largely free of electrolytes, is separated in the centrifuge and is partially dried by treatment with absolute acetone. Drying is continued for 5 to 10 minutes *in vacuo*. Some inert material is rendered insoluble, but the thrombin can be extracted with the use of about 40 cc. of water. It is important to break up all small particles. To the extract add acetic acid (about 3.2 cc. of 0.25 per cent) until the pH is 5.3 to 5.0. Centrifuge and discard the precipitate. There is some advantage in adding the acetic acid in three stages, and centrifuging after each addition. Coprecipitation apparently is reduced. From about pH 5.5 to 5.0 there is very little precipitation. The solution is then treated with acetone, as above, and dried *in vacuo*. A gray-white, amorphous, water-soluble product is obtained. The yield is 6 to 20 mg. from 1.5 liters of oxalated plasma.

Properties of Thrombin Preparation—The bioassay technique of Warner, Brinkhous, and Smith (4, 5) was used. 1 unit of thrombin is that amount which will clot 1 cc. of fibrinogen solution in 15 seconds. The clotting mixture contains 2 per cent acacia to provide colloid and to stabilize the clotting mechanism (4). It

contains 0.9 per cent NaCl. The pH of all reagents was adjusted to 7.1 to 7.3, and the temperature was 26–29°.

Apparently the dried thrombin retains its activity indefinitely. The same is true of thrombin solutions preserved at –35°. At 2°, 20 to 40 per cent of the activity is lost in 2 weeks. At room temperature bacterial growth may play a rôle in destroying thrombin. There is practically no loss in 12 hours, but within 3 days somewhat more than half of the activity disappears.

The thrombic activity of different preparations varies somewhat from one case to another, but the variations are not extreme. The most potent preparation thus far obtained showed 540 thrombin units per mg. of dry material. Values of 300 to 500 units per mg. are usually obtained. Using the assay method of Mellanby (1), we find that 0.0018 mg. of our most potent preparation clots 1 cc. of oxalated beef plasma in 30 seconds. The product is thus about 5 times as potent as that obtained by Mellanby.

The oxalated beef plasma from which our thrombin is prepared contains large amounts of oxalate solution, and because of this dilution the potential thrombin obtainable is only about 200 units per cc. of plasma. From each liter of this plasma we obtain about 4 to 15 mg. of purified thrombin, indicating a yield of about 1 to 5 per cent.

The most highly potent preparation of prothrombin thus far prepared in this laboratory has a titer of 151 units per mg. of dry material. Dry thrombin prepared from it contains 300 to 540 equivalent units per mg. One might assume from this that there is cleavage of the prothrombin molecule when thrombin forms. On the other hand, prothrombin is brought down in the precipitate which forms at pH 5.3, and no doubt impurities accompany it. These impurities are eliminated when thrombin is treated in this way, for thrombin is soluble at this pH.

SUMMARY

A procedure is described for the preparation of thrombin from ox plasma. Prothrombin is first prepared by the method previously described. It is converted into thrombin by the addition of calcium and thromboplastin which has been freed of antithrombin. For purification, the thrombin mixture is largely freed of electrolytes by acetone precipitation and drying. Thrombin is

then extracted from the precipitate with water. Impurities are precipitated from the aqueous solution with acetic acid. Thrombin is then precipitated and dried with acetone. The final product is a gray-white amorphous powder which is highly soluble in water. It contains 300 to 540 thrombin units per mg. of dry material.

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HEPATIC KETOGENESIS AND KETOLYSIS IN DIFFERENT SPECIES

BY PHILIP P. COHEN AND IRENE E. STARK

(From the Department of Physiological Chemistry, University of
Wisconsin, Madison)

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It is generally agreed that in the animal body the liver is the chief site of ketogenesis (1-4). Ketolysis, on the other hand, has been claimed to be chiefly an extrahepatic function (3-9).

Jowett and Quastel (4) reported that rat liver slices were capable of destroying ketone bodies under aerobic conditions, an effect which they found was inhibited by malonic acid. This finding has been confirmed by Edson and Leloir (7) and Stark and Cohen (10).

The monkey, as well as man, develops a ketosis rapidly and readily in contrast to many other species, such as the rat, rabbit, and guinea pig. We were led to investigate the rates of hepatic ketogenesis and ketolysis in the well fed and fasted rat, rabbit, guinea pig, and monkey as a possible basis for explaining this species difference.

Methods

Liver slices from healthy well fed and fasted male white rats, rabbits, guinea pigs, and *Macacus rhesus* monkeys were used in a Barcroft-Warburg apparatus. The fasting period was 24 hours except in the case of the rabbits which were fasted for 36 hours. The liver slices, 10 to 20 mg. of dry weight, were immersed in a Ringer-phosphate buffer solution of pH 7.4, to which was added the substrate previously neutralized to the same pH. The flasks were filled with pure oxygen and shaken for 1 hour at 38°.

Sodium butyrate made up to give a 0.01 M concentration was used as a substrate for determining the rate of ketogenesis. Sodium acetoacetate, prepared according to Ljunggren (11), and freed of methyl alcohol, was used as substrate in determining the

rate of ketolysis. Acetoacetic and β -hydroxybutyric acids were determined at the end of the experimental period.

Edson's (12) method of filtrate preparation and acetoacetic and β -hydroxybutyric acid determinations was used with slight modifications. Control experiments, to which no substrate was added, were run in all cases and treated similarly to the non-control experiments. Sodium acetoacetate solutions were freshly prepared on the day previous and the strength of the solution determined in quadruplicate on the day it was used as substrate. The concentration of acetoacetic acid used as substrate was between 0.006 and 0.008 M in the different experiments. Liver glycogen determinations, by the combined methods of Good, Kramer, and Somogyi (13) and Somogyi (14), were made in order to check the nutritional state of the animals.

Procedure

Four differential manometers and eight flasks were set up to permit measuring the rate of ketogenesis, ketolysis, spontaneous ketogenesis (no substrate added), and oxygen consumption, liver slices from a single animal being used.

At the end of 1 hour of shaking at 38° the flasks were removed from the water bath and placed in an ice bath for 20 minutes to insure condensation of volatile products. The liver slices were then removed and washed thoroughly with fine streams of distilled water. The washings and the flask contents were combined and treated with $\text{Ca}(\text{OH})_2$ and CuSO_4 to give a protein- and carbohydrate-free filtrate. Acetoacetic and β -hydroxybutyric acids were determined on these filtrates.

Units

ΔQO_2	= c.mm. of oxygen consumed per mg. of tissue (dry weight) per hour in the experimental flask (substrate present) over an equal weight of tissue in the control flask (no substrate present)
QAcOAc	= c.mm. of CO_2 equivalent to acetoacetic acid formed per mg. of tissue (dry weight) per hour
QBOH	= c.mm. of CO_2 equivalent to β -hydroxybutyric acid formed per mg. of tissue (dry weight) per hour
QKet	= sum of QAcOAc and QBOH
$\text{QAcOAc (theoretical)}$	= c.mm. of CO_2 equivalent to acetoacetic acid added initially per mg. of tissue (dry weight)
$-\text{QAcOAc}$	= difference between $\text{QAcOAc (theoretical)}$ and $\text{QKet at end of 1 hour}$

Results

In this study seven guinea pigs, ten rats, six rabbits, and three monkeys were used. Tables I through IV are combined protocols of typical experiments on a well fed and fasted animal of each species. Tables V to VII represent a compilation of the averages of all experiments.

The rate of spontaneous ketogenesis (no substrate added) in fasted and well fed animals is seen in Table V. It is apparent that

TABLE I

Combined Data from Typical Experiments on Hepatic Ketogenesis and Ketolysis in Well Fed and Fasted Rat

The final concentration of butyrate was 0.01 M; of acetoacetate, 0.007 M.

Substrate	Condition of animal	ΔQO_2	QAcOAc	QBOH	QKet	QAcOAc (theoretical)	-QAcOAc
Butyrate	Well fed	-3.91	3.57	4.24	7.81		
			3.40	4.70	8.10		
	Fasted	-3.04	5.88	2.92	8.80		
			6.20	3.10	9.30		
Acetoacetate	Well fed	+0.65	12.40	4.33	16.73	19.75	3.02
			19.25	4.00	23.25	27.44	4.19
			15.05	3.70	18.75	21.00	2.25
	Fasted	-0.84	13.15	6.40	19.55	20.70	1.15
			23.40	5.40	28.80	29.91	1.11
			15.10	7.16	22.26	22.70	0.44
None	Well fed		0.78	1.36	2.14		
			0.90	2.25	3.15		
	Fasted		1.35	2.49	3.84		
			1.37	2.85	4.22		

fasting results in a considerable increase in ketone body formation in all four species.

The rate of ketogenesis from butyric acid in fasted and well fed animals is seen in Table VI. In all four species there was an apparent higher rate of ketogenesis in the case of the fasted animals, although the increase is marked only in the case of the rabbit. This may be related to the fact that the rabbits were fasted for 36 hours, whereas the other species were fasted 24 hours.

Edson (12) has reported increases in QAcOAc of approximately

double the normal with liver slices from fasted rats in the presence of butyric acid. Jowett and Quastel (15) on the other hand have reported slight but significant increases in QAcOAc in the case of liver slices from fasted guinea pigs in the presence of butyric acid.

It is to be noted that the QBOH values in all instances are approximately equal to the QAcOAc values. This is in confirmation of Jowett and Quastel (16) and emphasizes the need for

TABLE II

Combined Data from Typical Experiments on Hepatic Ketogenesis and Ketolysis in Well Fed and Fasted Guinea Pig

The final concentration of butyrate was 0.01 M; of acetoacetate, 0.006 M.

Substrate	Condition of animal	ΔQO_2	QAcOAc	QBOH	QKet	QAcOAc (theoretical)	-QAcOAc
Butyrate	Well fed	-2.57	1.83	2.09	3.91		
			1.86	1.73	3.59		
	Fasted	-1.91	2.28	2.32	4.60		
Acetoacetate	Well fed	+0.19	1.33	3.13	4.46		
			6.95	2.69	9.64	12.29	2.65
			7.35	2.69	10.04	12.68	2.64
			7.38	2.41	9.79	11.27	1.48
	Fasted	-0.28	7.46	4.07	11.53	14.36	2.83
			13.80	3.72	17.52	17.85	0.33
			12.70	4.36	17.06	17.92	0.86
			11.65	3.98	15.63	16.80	1.17
			11.25	2.93	14.18	14.65	0.47
None	Well fed		0.44	0.67	1.11		
			0.84	0.76	1.60		
	Fasted		1.46	1.73	3.19		
			1.20	1.56	2.76		

determining both QBOH and QAcOAc where a measure of ketogenesis is desired.

The ΔQO_2 values for rat and guinea pig liver in the presence of butyrate are in agreement with those previously reported by Cohen (17), Edson (12), and Jowett and Quastel (15, 16).

The rate of hepatic ketolysis in fasted and well fed animals is seen in Table VII. It is seen from these data that the well fed rat, rabbit, and guinea pig have a considerable capacity for de-

TABLE III

Combined Data from Typical Experiments on Hepatic Ketogenesis and Ketolysis in Well Fed and Fasted Rabbit

The final concentration of butyrate was 0.01 M; of acetoacetate, 0.007 M.

Substrate	Condition of animal	ΔQO_2	QAcOAo	QBOH	QKet	QAcOAo (theoretical)	-QAcOAo
Butyrate	Well fed	-2.17	2.72	2.82	5.54		
			2.91	3.30	6.21		
	Fasted	-4.32	3.80	4.06	7.86		
			4.29	3.15	7.44		
Acetoacetate	Well fed	-0.52	4.89	3.98	8.87	13.50	4.63
			4.82	3.73	8.55	12.58	4.03
			3.92	4.77	8.69	14.30	5.61
			5.70	4.40	10.10	13.32	3.22
	Fasted	-0.90	11.10	2.73	13.83	14.90	1.07
			11.03	2.50	13.53	14.55	1.02
			0.74	0.28	1.02		
			0.73	0.45	1.18		
None	Well fed		0.74	0.28	1.02		
			0.73	0.45	1.18		
	Fasted		1.54	1.17	2.71		
			1.67	1.09	2.76		

TABLE IV

Combined Data from Typical Experiments on Hepatic Ketogenesis and Ketolysis in Well Fed and Fasted Monkey

The final concentration of butyrate was 0.01 M; of acetoacetate, 0.007 M.

Substrate	Condition of animal	ΔQO_2	QAcOAo	QBOH	QKet	QAcOAo (theoretical)	-QAcOAo	
Butyrate	Well fed	-4.57	2.94	4.09	7.03			
			2.72	3.01	5.83			
	Fasted	-4.45	3.16	4.39	7.55			
			2.92	4.42	7.34			
Acetoacetate	Well fed	-0.10	12.80	4.12	16.92	17.75	0.83	
			14.75	1.53	16.28	16.80	0.52	
	Fasted	-0.40	6.32	5.63	11.95	11.80	+0.15	
			5.97	5.36	11.33	11.86	0.53	
			5.56	5.82	11.38	11.65	0.27	
			6.95	4.90	11.85	11.60	+0.25	
	Well fed		1.21	0.77	1.98			
			1.14	1.33	2.47			
None	Fasted		0.90	2.07	2.97			
			1.10	2.24	3.34			

TABLE V
Spontaneous Ketogenesis (No Substrate)

	QAcOAc	QBOH	QKet
Normal rat.....	0.88	1.52	2.40
Fasted ".....	1.83	2.19	4.02
Normal rabbit.....	0.76	0.62	1.38
Fasted ".....	1.51	1.02	2.53
Normal guinea pig.....	0.74	0.58	1.28
Fasted " ".....	1.43	1.32	2.75
Normal monkey.....	1.17	1.05	2.22
Fasted ".....	1.00	2.13	3.13

TABLE VI
Ketogenesis from Butyric Acid (0.01 M)

	ΔQO_2	QAcOAc	QBOH	QKet
Normal rat.....	-4.66	4.10	4.27	8.37
Fasted ".....	-3.07	5.10	4.07	9.17
Normal rabbit.....	-2.61	2.78	2.81	5.59
Fasted ".....	-3.76	4.54	3.45	7.99
Normal guinea pig.....	-2.35	1.99	1.82	3.81
Fasted " ".....	-1.91	2.14	2.26	4.40
Normal monkey.....	-4.57	2.83	3.55	6.38
Fasted ".....	-4.45	3.04	4.40	7.44

TABLE VII
Ketolysis of Acetoacetic Acid (0.0060 to 0.0080 M)

	ΔQO_2	QBOH	-QAcOAc
Normal rat.....	+0.46	4.01	3.15
Fasted ".....	-1.11	7.28	0.80
Normal rabbit.....	-0.43	3.88	3.92
Fasted ".....	-0.65	3.27	0.65
Normal guinea pig.....	+0.19	3.65	2.30
Fasted " ".....	-0.28	3.75	0.63
Normal monkey.....	-0.10	3.07	0.68
Fasted ".....	-0.40	5.43	0.10

stroying acetoacetate other than by conversion to β -hydroxybutyric acid. The percentage destruction in these instances is of the order of 14, 25, and 20, respectively, for the above three

species. These values are in reasonable agreement with those of Snapper and Grünbaum (6) for perfused dog liver, which showed about 15 per cent destruction. The $-QAcOAc$ values for rat and guinea pig are considerably higher than those reported by Edson and Leloir (7) and Quastel and Wheatley (8).

The most striking thing in these data is the inability of liver slices from a well fed monkey to destroy appreciably acetoacetic acid.

In the case of liver slices from fasted animals, there is a marked decrease in ketolytic activity with the rat, rabbit, and guinea pig, while monkey liver shows but a slight decrease. It should be noted, however, that liver slices from *fasted* rats, rabbits, and guinea pigs show a capacity for ketolysis about equal to that of liver slices from a *well fed* monkey.

The ΔQO_2 values for liver slices in the presence of acetoacetate are very low and in two instances are actually positive. The $+\Delta QO_2$ values imply that the rate of oxygen consumption by liver slices in the presence of acetoacetate is less than that of an equal weight of liver slices without any added substrate. Stöhr (18) has reported a decrease in QO_2 values in rat liver and kidney slices in the presence of acetoacetate. Earlier work by Wigglesworth (19) showed an increase in oxygen consumption by liver slices in the presence of acetoacetate. He interpreted this to mean an oxidative destruction of acetoacetate. While Stöhr (18) could not establish a significant difference between the oxygen consumption of liver slices from well fed and fasted rats in the presence of acetoacetate, it would appear from Table VII that there is a small increase in ΔQO_2 with liver slices from the fasted animals as compared with the normals. The increase appears to be marked only in the case of the fasted rat.

Table VIII shows the effect of fasting on ketogenesis and ketolysis. The values are the differences between the normal and fasted animals. It appears from Table VIII that in the case of the rat and guinea pig there is a greater increase in ketogenesis on fasting in the absence of substrate than in the presence of butyrate. This would imply that liver slices from fasted rats and guinea pigs oxidize less butyric acid than those of normal animals. The lowered ΔQO_2 (Table VI) values in these instances are in keeping with this. It is possible that the rapid production of

ketone bodies by spontaneous ketogenesis in these fasted animals has an inhibitory effect on the butyric acid oxidation. It is to be noted that the liver slices from the fasted rabbit on the other hand oxidize more butyric acid than the normal. The ΔQO_2 is also increased in this case (Table VI). The difference in the ability of liver slices from fasted rabbits, as compared to fasted rats and guinea pigs, to oxidize butyric acid may be due to the longer fasting period in the case of the rabbit. It is conceivable that the liver of the rabbit fasted for 36 hours has used up a larger portion of its metabolites including fat. This is supported by the lower level of spontaneous ketogenesis of the fasted rabbit as compared with the fasted rat and guinea pig (Table V). It follows that butyric acid when added to liver slices from the fasted rabbit will be more readily oxidized both because of the depletion of other

TABLE VIII
Effect of Fasting on Ketogenesis and Ketolysis

	ΔQ_{Ket} (no substrate)	ΔQ_{Ket} (butyrate)	ΔQ_{AcOAc}
Rat.....	+1.62	+0.80	-2.35
Rabbit.....	+1.15	+2.40	-3.27
Guinea pig.....	+1.47	+0.59	-1.67
Monkey.....	+0.91	+1.06	-0.58

substrates and the presence of a smaller concentration of spontaneously produced ketone bodies, which act as an inhibitor of the butyrate oxidation.

In the case of the monkey, the rate of ketogenesis in the presence of butyrate is of about the same order as the rate of spontaneous ketogenesis in the fasted animal. Thus in this case butyrate is oxidized at about the same rate. The ΔQO_2 values are in support of this (Table VI).

The decrease in ketolytic power on fasting is seen in the third column of Table VIII. It is apparent that with the exception of the monkey, the decrease in ketolytic capacity on fasting is of such a magnitude as to account for more than the increase in ketogenesis. It is of interest to note that the greatest decrease in rate of ketolysis with fasting (rabbit) is associated with the greatest increase in ketogenesis from butyric acid. In the case

of the monkey, the decrease of ketolysis with fasting is slight as would be expected from the fact that the normal liver has but a very limited ketolytic capacity.

Table IX gives glycogen values for the well fed and fasted rat and monkey. It is to be noted that at the time of introduction to the flasks, the glycogen value of the liver slices from the well fed monkey is quite high (3.20 per cent). Thus the failure of normal monkey liver to break down acetoacetate cannot be due to a low liver glycogen.

TABLE IX
Per Cent Glycogen in Rat and Monkey Liver

	Normal		Fasted	
	Rat	Mon-key	Rat	Mon-key
Immediately on death.	4.09	5.09	0.55	0.37
At time of introduction of slices to flasks, usually 45 to 60 min. after death.	1.97	3.20	0.43	0.33

DISCUSSION

Quastel and Wheatley (8) and Edson and Leloir (7) using liver slices from normal animals have reported much lower rates of hepatic ketolysis than those reported here. The rates reported by these workers in the case of normal rat and guinea pig are of the order reported here for fasted animals. A possible explanation for the differences resides in the fact that these workers employed a bicarbonate-buffer system in their experiments, whereas a phosphate-Ringer's solution buffer system was employed by us. Ciaranfi (20) studying the metabolism of butyric acid and ketone bodies in guinea pig liver slices reported that in the presence of phosphate-Ringer's solution there is a considerably greater oxidation of butyric acid and breakdown of acetoacetate than in the presence of bicarbonate-Ringer's solution. On this basis one might speculate that a phosphorylation mechanism may be involved in hepatic ketolysis.

The failure of liver slices from a well fed monkey to destroy ketone bodies to any appreciable extent, as compared with the other three species, is difficult to explain on any other basis than

a species difference. It is well known that there is a marked difference between Primates and the lower species in their susceptibility to a ketosis (21-23). Whether or not this difference in hepatic ketolysis is part of the explanation for the difference in susceptibility to a ketosis between the species is difficult to say. The large amount of work with various techniques showing the extrahepatic tissues, especially the muscles and kidneys (3-9), to be much more active ketolytically than the liver is very convincing. However, it is conceivable that hepatic ketolysis can keep pace with hepatic ketogenesis, within broad limits in the case of the rat, rabbit, and guinea pig, and very narrow limits in the case of the monkey, and so serve to keep the level of ketonemia low. The lowering of liver glycogen, as by fasting, would result early in a ketonemia in the case of the monkey, and only late in the case of the other three species, because of the differences in their capacity for hepatic ketolysis.

The relationship between fasting and hepatic ketolysis would seem to implicate liver glycogen as the most important factor. From the data presented here, it would appear that the increase in hepatic ketogenesis (spontaneous) with fasting is more than accounted for by a failure in hepatic ketolysis. The metabolic relationship between hepatic glycogen and ketolysis, however, is still obscure. The mechanism of ketolysis, especially in the kidney, has been studied by Edson and Leloir (7) and Quastel and Wheatley (8). The possible rôle of the succinate system in the mechanism has been considered by both groups of the above workers but there is no basis as yet for stressing the importance of this system in explaining the ketolytic mechanism. The observed action of malonate on inhibiting the breakdown of acetoacetate (4, 7, 10) is probably not entirely due to an inhibition of the succinate system. It is to be hoped that further investigations on the intermediate stages of the ketolytic mechanisms will bring out the relationship between glycogen and ketolysis more clearly.

SUMMARY

1. Liver slices from a well fed monkey have a very low rate of ketolysis as compared with liver slices from well fed rats, rabbits, and guinea pigs.

2. Liver slices from *fasted* rats, rabbits, and guinea pigs have very low rates of ketolysis, of about the same order as liver slices from a *well fed* monkey. Fasting in the case of the monkey results in a slightly lower rate of ketolysis.

3. The difference between the rates of hepatic ketolysis of the monkey and the other three species is discussed with relation to the differences in susceptibility to ketosis between the Primates and the lower species.

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STUDIES ON PANCREATIC LIPASE

III. THE ACTIVATION OF THE WASHED ENZYME BY BLOOD SERUM AND OTHER SUBSTANCES

By L. RABINOWITCH AND A. M. WYNNE

*(From the Department of Biochemistry, University of Toronto,
Toronto, Canada)*

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The question as to the existence of a coenzyme which is necessary for the activity of pancreatic lipase has, for many years, been a controversial one. Nearly 30 years ago Rosenheim (1) made certain observations which led him to suggest that such a coenzyme may exist. By diluting a crude aqueous extract of pig pancreas with water he obtained a precipitate which was inactive toward olive oil but which acquired lipolytic power after the addition of blood serum or a boiled and filtered portion of the original extract. Later investigators, including Umeda (2), Shaw-Mackenzie (3), Corran (4), Corran and Lewis (5), and Woodhouse (6), have studied the activation of similar precipitates. Umeda's work pointed very strongly to the conclusion that the activations which he and previous workers observed were due merely to the buffering action of the added substances; subsequent workers, however, have very largely failed to explore this possibility, although Fine (7) has emphasized the probability that the influence of serum on enzymic activity is chiefly a function of its buffering capacity. Woodhouse, on the other hand, attributes the activating effects of sera, urine, and ovarian fluid on "prolipase," *i.e.* the washed enzyme, to the presence of a coenzyme in these substances.

Since this use of the term coenzyme as applied to the pancreatic lipase system has been incorporated in the general enzyme literature (Tauber (8)), an attempt has been made in the present work (1) to determine whether there exists, in the case of this lipolytic system, a specific and indispensable activator or coenzyme, and (2) to elucidate the cause of the activations which

undoubtedly occur when substances such as serum and boiled enzyme are added to the prolipase. The results indicate that pancreatic lipase does not require a specific coenzyme and that the observed activations are due mainly to the buffering capacities of the added substances.

Methods and Results

Enzyme Preparation—The following method is based on that of Woodhouse (6). 1 volume of minced pig pancreas was shaken with 2 volumes of distilled water for 2 hours at room temperature; the mixture was filtered through cheese-cloth and centrifuged at 1500 R.P.M. for 3 minutes. The supernatant fluid was found to be very active lipolytically. To prepare prolipase, 1 volume of enzyme solution was diluted with 9 volumes of distilled water and several such dilutions were acidified with varying amounts of lactic acid; the pH of the different mixtures was measured and, after standing for 1 hour, the precipitates were separated and weighed. The greatest precipitation occurred at pH 2.9 to 3.0; the curve correlating pH and amount of precipitate dropped sharply on both sides of this optimum. Prolipase preparations were found by Woodhouse to be only slightly active toward olive oil; we have confirmed this result but have observed quite marked activity toward tripropionin. By washing the precipitate several times with water this activity could be considerably reduced, but in no case was it possible, even after several washings, to obtain a preparation which was completely inactive toward tripropionin.

Preparation of Digestion Mixtures—Each digestion mixture contained 10 cc. of substrate emulsion, 1 cc. of washed enzyme (prolipase) solution, activator or other substance under investigation, and CO₂-free water to make a total volume of 50 cc. The substrate most frequently used was tripropionin; an emulsion was prepared by shaking 10 cc. (*i.e.* 10.8 gm.) of tripropionin in 50 cc. of 5 per cent gum acacia solution. When diluted to 50 cc. in the digestion mixture 10 cc. of this emulsion provided a concentration of tripropionin equivalent to 3.6 per cent.

The analytical procedure was essentially the same as that described by Weinstein and Wynne (9). The titration figures in Tables I to VI represent the amount (cc.) of 0.05 N acid liberated in 50 cc. of digestion mixture.

Activation of Washed Enzyme—Preliminary experiments were conducted without reference to pH; the effects of added substances are illustrated by the results in Tables I and II. It should be stated that in the two sets of experiments, represented by Tables I and II, different enzyme preparations were used. The term

TABLE I

Hydrolysis of 3.6 Per Cent Tripropionin Emulsion by Washed Lipase (First Preparation) in Presence and Absence of Added Substances

Time	0.05 N acid liberated in 50 cc. digestion mixture			
	Washed enzyme	Washed enzyme plus		
		1 cc. enzyme dialysate	1 cc. deaminated enzyme dialysate	1 cc. ox serum
min.	cc.	cc.	cc.	cc.
5	1.80	2.80	2.50	2.75
10	3.20	4.80	4.40	5.10
15	4.20	6.55	6.05	6.75

TABLE II

Hydrolysis of 3.6 Per Cent Tripropionin Emulsion by Washed Lipase (Second Preparation) in Presence and Absence of Added Substances

0.05 N acid liberated in 50 cc. digestion mixture

Time	Washed enzyme plus						
	Washed enzyme	2 cc. enzyme dialysate	2 cc. glutamic acid solution (1 per cent)	2 cc. glutamic acid solution (1 per cent) deaminated and neutralized	2 cc. phenylalanine solution (1 per cent)	2 cc. phenylalanine solution (1 per cent) deaminated and neutralized	5 cc. 0.05M phosphate buffer (pH 6.8)
min.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
5	2.25	3.25	3.35	2.20	5.65	0.80	4.00
10	4.10	7.00	6.85	4.05	9.95	1.40	7.50
15	5.40	10.25	10.35	5.35	13.75	1.90	10.75

enzyme dialysate refers to a solution obtained in the following manner. A fresh, active, aqueous extract of the gland was boiled and filtered; the filtrate was dialyzed against distilled water for three 8 hour periods, fresh distilled water being used each time. The total dialysate was concentrated at low temperature to a

volume equivalent to that of the original enzyme solution. The dialysate, as finally used, contained 7.05 mg. of inorganic phosphorus and 7.1 mg. of amino nitrogen per cc.; its activating effect was found to be equivalent to that of the boiled and filtered enzyme solution before dialysis. The results (Tables I and II) show that each of the substances, enzyme dialysate, ox serum, glutamic acid, phenylalanine, and phosphate buffer (pH 6.8), increased the activity of the washed enzyme toward tripropionin. Deamination of the two amino acids destroyed their activating power; deamination of the enzyme dialysate destroyed less than 25 per cent of its activating power.

To test the effect of phenylalanine on the hydrolysis of cod liver oil by the washed enzyme three digests were incubated for 48 hours, after which the liberated acid was titrated. The initial concentration of the emulsified oil in each digest was 3.1 per cent; the other conditions were identical with those of previous experiments. The results reveal an effect of phenylalanine comparable with that observed by Woodhouse in the prolonged hydrolysis of olive oil in the presence of added serum or boiled enzyme.

0.05 N acid liberated in 50 cc. digest		
Washed enzyme	Washed enzyme + 2 cc. phenylalanine solution (1 per cent)	2 cc. phenylalanine solution (1 per cent), no enzyme
2.00	27.80	1.50

Hydrolysis of Tripropionin in Buffered Mixtures—The pH of four digestion mixtures, prepared as before except for the addition of buffer, was adjusted to 6.8 by adding phosphate buffer. The results in Table III show that the initial velocities of hydrolysis were unaffected, under the conditions of the experiment, by the filtrate from boiled enzyme and by ox serum. Egg albumin had a slight retarding effect.

Change of pH in Absence and in Presence of Activator—Two 50 cc. digests were allowed to run for 48 hours: one contained washed enzyme and tripropionin emulsion; the other contained, in addition, 1 cc. of enzyme dialysate. At the times indicated in Table IV the total acid and pH were determined.

At the apparent isoelectric point of the washed enzyme (pH 2.9 to 3.0) the activity of the system is very much reduced and at pH levels more acid than this it becomes almost negligible. The results in Table IV show that the effects of the enzyme dialysate are (1) to establish a less acid initial pH closer to the optimum

TABLE III

Hydrolysis of 3.6 Per Cent Tripropionin Emulsion at pH 6.8 (Phosphate Buffer) in Presence and Absence of Added Substances

	0.05 N acid liberated in 50 cc. digestion mixture	
	5 min.	10 min.
	cc.	cc.
Washed enzyme alone (1 cc.)	2.95	4.65
" " + filtrate from boiled enzyme (1 cc.) . .	3.05	4.70
" " + ox serum (1 cc.)	3.00	4.60
" " + 2% egg albumin solution (1 cc.) . . .	2.55	4.00

TABLE IV

Buffering Action of Enzyme Dialysate in Hydrolysis of Tripropionin

Time	Washed enzyme		Washed enzyme + enzyme dialysate	
	0.05 N acid liberated in 50 cc.	pH	0.05 N acid liberated in 50 cc.	pH
min.	cc.		cc.	
0		4.75		5.95
10	2.50		5.95	
30	5.30		9.75	
hrs.				
1	7.10		14.75	
24	16.50	3.05	33.50	3.20
31	17.50	3.05	36.25	3.15
48	17.50	3.05	37.00	3.05

value, which is about pH 7 when phosphate buffer is used, and (2) to delay the time at which the acidity of the mixture reaches a level corresponding to pH 3.0 at which the activity is greatly diminished. A similar buffering effect of ox serum was observed in an experiment whose results are summarized in Table V.

TABLE V
Hydrolysis of Olive Oil

	By washed en- zyme alone (1 cc.)	By ox serum alone (1 cc.)	By washed en- zyme (1 cc.) + ox serum (1 cc.)
0.05 N acid liberated in 50 cc. diges- tion mixture in 48 hrs., cc	4.90	3.90	20.80
Initial pH	3.55		4.55
pH after 48 hrs.	3.1	4.6	3.1

TABLE VI
*Comparative Activating Powers of Sera and Other Substances in Standard
Tripropionin Digests*

Activator (1 cc.)	0.05 N acid liberated in 72 hrs. cc.
Control 1. No enzyme, 1 cc. ox serum	4.80
" 2. " activator	16.30
Human serum	32.30
Enzyme dialysate	30.70
Horse serum	29.50
Rabbit serum	28.10
Ox serum	26.25
Guinea pig serum	25.60
Chicken plasma	25.00
Dog serum	22.50

TABLE VII
Comparative Buffering Capacities of Sera and Other Substances

Added substance (1 cc.)	pH of solution (50 cc.) of 0.04 N propionic acid
Without added substance	2.97
Enzyme dialysate	3.30
Rabbit serum	3.25
Human "	3.22
Horse "	3.17
Guinea pig serum	3.16
Chicken plasma	3.16
Ox serum	3.15
Dog serum	3.15

Comparative Effects of Different Activators in Relation to Their Buffering Capacities—In Table VI are shown the amounts of acid liberated in 72 hours in standard tripropionin digests to which were added 1 cc. portions of serum or other activator. Of the various sera examined, human serum had the greatest and dog serum the least activating effect. The buffering capacities of the substances were tested by measuring the pH of a series of solutions of propionic acid, each containing 1 cc. of activator; results were obtained of which those in Table VII are typical. The pH differences in several cases are insignificant but one can observe a general correlation between the order of the buffering capacities (Table VII) and that of the activating powers (Table VI) of the substances; enzyme dialysate, and human, rabbit, and horse sera occupy the top positions in both Table VI and Table VII.

SUMMARY

It is concluded that the activation of washed pancreatic lipase by blood sera and by the dialysate from boiled enzyme is largely due to their buffering capacities. A similar activation is observed on adding phosphate buffer or a single amino acid such as phenylalanine or glutamic acid. Destruction of the amphoteric character of the amino acids destroys their activating powers. The activating power of the thermostable substances in the enzyme dialysate is only partially destroyed by nitrous acid treatment; the greater part of the activating power of such preparations is probably due to the buffering action of phosphates. The use of the term coenzyme to describe activators of this type is not justifiable, since there is no evidence to show that they are specific and indispensable.

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THE EFFECT OF SUPPLEMENTARY LYSINE, METHIONINE, AND CYSTINE ON THE PRODUCTION OF FATTY LIVERS BY HIGH FAT DIETS CONTAINING GLIADIN

By HELEN F. TUCKER* AND HENRY C. ECKSTEIN

*(From the Department of Chemistry, Skidmore College, Saratoga Springs,
and the Department of Biological Chemistry, Medical School,
University of Michigan, Ann Arbor)*

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In a recent paper (1), it was shown that the total lipid content of rat livers was 41 per cent less when a diet containing 5 per cent casein, 40 per cent lard, and 0.5 per cent methionine was fed than when the methionine was omitted. When the added methionine was replaced by cystine, the total lipid content of the rat livers was 57 per cent higher than when neither methionine nor cystine was added. The two sulfur-containing amino acids thus appeared to exert opposite effects on the deposition of fat in the livers of rats fed diets low in casein and high in fat. In this connection, it is to be noted that Best, Grant, and Ridout (2) have reported that a diet containing 10 per cent casein, 20 per cent gelatin, and 40 per cent beef drippings produced fatty livers in rats, whereas a similar diet containing 30 per cent casein and no gelatin did not. In view of the fact that gelatin contains relatively little methionine (3), the results of these investigations are now explainable on the basis of the low methionine content of the casein-gelatin diet and the higher methionine content of the casein diet containing the same percentage of total protein but no gelatin.

It seemed of interest to investigate further the effect of dietary methionine and cystine on liver fat when these amino acids sup-

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plemented another type of protein. Gliadin was chosen because it is deficient in an amino acid. Casein is also inadequate for growth at the level fed (5 per cent), and its nutritive value for growth can be effectively increased by the addition of either cystine or methionine. Similarly, gliadin can be made more adequate for growth by the addition of lysine to the diet. Thus, it is not only possible to study the effects of cystine and methionine which do not improve the nutritive value of gliadin for growth, but also the effect of supplementary lysine which renders the protein more adequate for growth. The dihydrochloride of *dl*-lysine was used in place of the free amino acid. The gliadin was prepared accord-

TABLE I
Experimental Diets

All diets contained 2 per cent agar, 40 per cent lard, and 5 per cent salt mixture (5) in addition to the ingredients listed in the table. Each animal received one dried yeast tablet (400 mg.) and 1 drop of cod liver oil daily.

Diet No.	Gliadin	Glucose	<i>l</i> -Cystine	<i>dl</i> -Methionine	<i>dl</i> -Lysine dihydrochloride	Cystine in 100 gm. diet	Methionine in 100 gm. diet
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>
1	18	35				493	365
2	5	48				137	102
3	18	33.37			1.63	493	365
4	5	47.5	0.5			637	102
5	5	46.23			1.77	137	102
6	5	45.65		0.58	1.77	137	682

ing to the method of Nolan and Vickery (4). Analysis of the product obtained showed it to contain 13.8 per cent N calculated on the ash- and moisture-free basis.

Male rats weighing approximately 150 to 175 gm. were used in all experiments, the duration of which was 17 to 18 days. The diets are shown in Table I, which also includes the contents of cystine and methionine in mg. per 100 gm. of food, as calculated on the basis that gliadin contains 2.03 per cent methionine and 2.74 per cent cystine (6). The diets were essentially the same as those used in the previous investigation (1), except that gliadin was substituted for casein, and two levels of protein, 18 per cent (Diets 1 and 3) and 5 per cent (Diets 2, 4, 5, 6), were used. 0.58

per cent of *dl*-methionine was added to Diet 6 in order to raise the total methionine content to the same level as that of the 5 per cent casein plus 0.5 per cent methionine diet previously used (1). 1.77 per cent of *dl*-lysine dihydrochloride was added to Diets 5 and 6 in order to make the lysine content equivalent to that of Diet 3, which contained 18 per cent gliadin and 1.63 per cent of *dl*-lysine dihydrochloride. The analytical procedures were identical with those employed in the previous investigation (1).

Table II gives the data for the individual animals, as well as the average results obtained by feeding the various diets. It is clear from a comparison of the results obtained when Diets 1 and 2 were fed that the lipid content of rat livers increased when the methionine content of the diet was lowered, even though the content of cystine was also decreased. Thus, on the 18 per cent gliadin diet, in which the calculated methionine content was 365 mg. per 100 gm. of food, the average fat content of the livers was 7.3 per cent, whereas in Diet 2 (5 per cent gliadin), in which the methionine content was 102 mg. per 100 gm. of food, the average fat content was 33.8 per cent.

Table II also shows that dietary lysine has little effect on the fat content of rat livers when the level of gliadin in the diet is either high (18 per cent) or relatively low (5 per cent). The average value for the rats on the unsupplemented 18 per cent gliadin ration was 7.3 per cent, as compared with 6.5 per cent for the 18 per cent diet supplemented with 1.63 per cent of *dl*-lysine dihydrochloride. At a 5 per cent level, the mean value was 33.8 per cent when the 5 per cent gliadin diet was supplemented with 1.77 per cent of *dl*-lysine dihydrochloride.

The remainder of the data in Table II is concerned with the effects produced by supplementing the 5 per cent gliadin diet with methionine plus lysine and with cystine alone. The results show again the lipotropic action of methionine. A change from a methionine content of 102 mg. per 100 gm. of food in Diet 4 to 682 mg. in Diet 6 produced a 77.5 per cent decrease in the fat content of the livers. Thus, the average value obtained when the diet contained supplementary methionine was 7.6 per cent, as compared with 33.8 per cent for the low gliadin diet without supplement. The influence of supplementary cystine was by no means similar to that observed in the previous investigation (1)

TABLE II

Total Lipid Contents of Livers of Rats on Gliadin Diets and Gliadin Diets Supplemented by Amino Acids

The letter immediately following the number indicates the series of experiments.

Diet No.	Rat No.	Change in weight	Average daily food intake	Liver		
				Weight	Per 100 gm. rat	Total lipids
		<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
1	1A	+8.5	8.4	6.7	3.7	6.8
	2A	-20.7	5.0	4.5	3.7	5.1
	3A	+1.3	7.6	7.0	4.6	10.0
	Average.....	-3.6	7.0	6.1	4.0	7.3
2	1B	-4.4	8.0	8.5	4.9	25.5
	2B	0.0	11.3	11.1	6.5	39.8
	3B	-2.1	10.5	8.7	5.0	22.0
	1C	+3.6	11.0	14.0	8.2	37.8
	2C	+1.0	11.4	10.5	7.0	37.3
	3C	+4.6	10.2	14.6	8.0	40.5
	1E	+10.1	11.3	9.1	5.2	34.5
	2E	+7.7	11.6	9.2	5.5	27.5
	3E	+3.1	10.6	13.7	8.3	35.1
	4E	+6.3	9.3	12.1	7.2	38.4
	Average.....	+2.5	10.4	11.1	6.6	33.8
3	5A	+10.7	8.5	5.7	3.2	7.2
	6A	+6.3	7.8	7.1	4.2	5.4
	7A	+17.2	10.0	7.3	3.5	6.8
	Average.....	+11.4	8.8	6.7	3.6	6.5
5	4B	-6.0	10.0	7.7	4.9	28.1
	5B	+5.0	10.9	11.9	7.1	42.3
	6B	+3.6	10.1	8.6	4.3	17.0
	4C	-3.1	9.7	11.4	7.4	38.6
	5C	-9.1	4.6	9.3	6.7	35.7
	6C	+2.2	12.1	13.3	7.3	41.7
	Average.....	-1.2	9.5	10.3	6.3	33.9

TABLE II—*Concluded*

Diet No.	Rat No.	Change in weight	Average daily food intake	Liver		
				Weight	Per 100 gm. rat	Total lipids
		<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
4	13C	-3.1	9.3	10.8	6.9	38.9
	14C	+4.5	8.4	12.9	8.1	34.3
	15C	-1.3	7.3	9.4	6.2	30.0
	16C	+5.4	11.0	10.4	6.0	26.0
	17C	-1.8	8.0	14.3	8.8	38.5
	18C	+7.8	9.3	12.6	7.6	37.8
	7D	0.0	9.7	11.7	7.2	39.5
	8D	+6.1	9.3	8.7	5.0	26.9
	9D	+11.7	10.6	13.7	7.6	38.8
	5E	+12.1	8.0	13.1	7.5	36.9
	6E	+13.0	11.1	10.5	5.8	30.1
	7E	+7.1	10.0	14.2	8.2	38.9
Average.....		+6.2	9.5	11.8	7.1	34.9
6	8C	+1.1	9.3	6.5	3.7	5.9
	9C	+1.7	10.8	6.5	3.7	6.2
	10C	+1.8	12.0	5.3	3.3	9.4
	12C	+1.9	9.4	7.5	4.6	6.7
	4D	-5.9	5.6	5.4	3.7	6.8
	5D	+6.7	10.0	6.7	3.8	8.7
	1D	+6.5	6.9	6.0	3.6	8.8
Average.....		+2.0	8.9	6.2	3.7	7.6

when casein was used. The average value for the rats receiving the 5 per cent gliadin diet supplemented by cystine was 34.9 per cent, as compared with 33.8 per cent in Diet 4, in which the addition of cystine was omitted. This represents a negligible increase. On the other hand, when casein was the source of dietary protein, a 0.5 per cent supplement of cystine produced a 57 per cent increase in the fat contents of the livers (1). The 5 per cent casein diet supplemented with 0.5 per cent of cystine contained 566 mg. of cystine and 177 mg. of methionine per 100 gm. of food, as compared with 637 mg. of cystine and 102 mg. of methionine in 100 gm. of the diet containing 5 per cent gliadin plus 0.5 per cent of cystine. If the effects of cystine and methionine are

quantitative, more fats should have been found in the livers of the rats on the gliadin diet supplemented with cystine, because, as noted above, such a ration contains more cystine and less methionine than when casein is supplemented with cystine. It is, of course, possible that other amino acids exert the same effects as cystine and methionine, and it is, therefore, evident that more information is needed.

After the completion of the experimental work and the preparation of this paper, Channon and coworkers reported experiments bearing directly on this problem. In confirmation of the work of Tucker and Eckstein (1), the formation of fatty livers could be prevented by supplementing a diet containing 5 per cent ovalbumin and 40 per cent beef drippings with 0.5 per cent methionine (7). In a second paper, they reported that the production of fatty livers could be prevented by supplementing a diet containing 8 per cent casein and 39 per cent beef drippings with proteins (8). The lipotropic action was found to vary with the supplementary protein employed. From their data it appears that this action was related to the methionine content of the supplements. These supplementary proteins may be arranged in the order of their effectiveness as follows: casein, ovalbumin, fibrin, gliadin, and gelatin. This effectiveness is roughly proportional to the methionine content of these proteins (6). However, ovalbumin, which contains somewhat more methionine than does casein, is slightly less effective in its lipotropic action than is casein.

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SUMMARY

1. The total lipid content of rat livers increased from 7.3 per cent to 33.8 per cent when the gliadin content of a high fat diet (40 per cent lard) was decreased from 18 to 5 per cent.

2. The addition of lysine to a high fat diet (40 per cent lard) containing either 5 or 18 per cent of gliadin had little, if any, effect on the fat content of rat livers.

3. The total lipid content of rat livers was 77.5 per cent less when a 5 per cent gliadin diet containing 40 per cent lard was supplemented with methionine than when the methionine was omitted.

4. The addition of cystine to a diet containing 40 per cent lard and 5 per cent gliadin had little, if any, effect on the total lipid contents of rat livers.

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AUCUBA MOSAIC VIRUS PROTEIN ISOLATED FROM DISEASED, EXCISED TOMATO ROOTS GROWN IN VITRO

By W. M. STANLEY

*(From the Department of Animal and Plant Pathology of The Rockefeller
Institute for Medical Research, Princeton)*

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Excised tomato roots can be grown for potentially unlimited periods of time in a liquid medium of known constitution (1). When roots for such cultures are obtained from plants systemically infected with tobacco mosaic or aucuba mosaic virus, they grow as readily as do healthy roots and, although the roots exhibit no obvious symptoms, it can be shown that the virus multiplies at a rate paralleling that of the host tissue (2). Such roots are free from chlorophyll and present, in this and other respects, a much simplified physiology as compared with that of the tissues usually employed as sources of virus. The increase of tobacco mosaic virus in tissues apparently free of chlorophyll has also been reported by Holmes (3). The virus is apparently unaltered by cultivation in isolated roots, for the symptom-complex produced in plants inoculated with virus that had been carried through twenty passages in such roots did not differ from that produced by the virus at the start of the experiment (2). This paper reports some results of investigation of certain properties of virus protein produced under these simplified conditions. Excised tomato roots diseased with aucuba mosaic virus and grown *in vitro* were examined for the presence of the virus protein of high molecular weight previously isolated from the leaves of diseased plants (4). The rapid and efficient method of differential centrifugation (5) was used and the properties of the protein of high molecular weight found in the diseased roots were compared with those of aucuba mosaic virus protein obtained from leaves. A comparison of the total nitrogen, protein nitrogen, and virus protein nitrogen con-

tents of the juice from the tomato roots grown *in vitro* with those of the juices from the leaves, stems, and roots of normal tomato plants and of diseased tomato and Turkish tobacco plants is given.

EXPERIMENTAL

Roots of tomato (*Lycopersicon esculentum*, Miller) infected with aucuba mosaic virus and maintained in culture *in vitro* by Dr. Philip R. White since December 9, 1933, were used in the present study. A clone of 100 roots was subcultured at weekly intervals and, through Dr. White's courtesy, the residual tissue from all cultures was collected between November, 1936, and March, 1937, immediately frozen, and kept in the frozen state until a total of 20 gm. of such tissue was accumulated. Leaf tissue for comparison was obtained from five tomato plants inoculated on March 5, 1937, with virus obtained from the root cultures. The plants were cut and frozen on March 23, 1937.

The 20 gm. mass of infected root tissue was put through a meat grinder while frozen, 2 cc. of a solution of 50 per cent K_2HPO_4 were added, and the pulp was allowed to thaw. The juice was expressed, analyzed for total and protein nitrogen, and tested for virus activity. The pulp was extracted with 20 cc. of 0.1 M phosphate buffer at pH 7, and this extract was combined with the juice, making a total of 33 cc. Cellular debris was removed from the combined extracts by centrifugation in a Swedish angle centrifuge for 30 minutes. The supernatant liquid was then ultracentrifuged for 90 minutes at a speed of about 24,000 R.P.M., corresponding to a force of about 60,000 times gravity. The supernatant liquid was removed, analyzed, and tested for virus activity (Preparation 1, Table I). The sediment in the bottoms of the tubes was suspended in 7 cc. of 0.1 M phosphate buffer at pH 7, subjected to low speed angle centrifugation, and the supernatant liquid removed and ultracentrifuged for 90 minutes. The sediment obtained on ultracentrifugation was again suspended in 7 cc. of 0.1 M phosphate buffer and subjected first to low speed centrifugation and then to ultracentrifugation. The material that was obtained following the third ultracentrifugation was dissolved in 5 cc. of 0.1 M phosphate buffer at pH 7. This solution was found to give the usual tests for protein and to contain a total of 1.7 mg. of protein. This yield of protein of high molecular

TABLE I
Yields of Virus Protein, Virus Activity, and Nitrogen Content of Juices from *Aucuba Mosaic-Diseased Tomato Roots Grown in Vitro* and from *Roots, Leaves, and Stems of Tomato and Turkish Tobacco Plants Diseased with Aucuba and Tobacco Mosaic Viruses*

Preparation*		Juices from leaves			Juices from stems			Juices from roots		
		Total N	Protein N	Virus activity	Total N	Protein N	Virus activity	Total N	Protein N	Virus activity
		mg. per cc.	mg. per cc.		mg. per cc.	mg. per cc.		mg. per cc.	mg. per cc.	
1. Aucuba-diseased tomato roots grown <i>in vitro</i>	Before ultracentrifugation Supernatant after first ultra-centrifugation									+ -
2. Aucuba-diseased tomato plants	Virus protein N isolated Before ultracentrifugation Supernatant after first ultra-centrifugation	0.47	0.32	+	-					0.02
3. Aucuba-diseased tomato plants	Virus protein N isolated Before ultracentrifugation Supernatant after first ultra-centrifugation	1.1	0.57	+	-	0.39	0.13	+	0.47	0.15
		0.84	0.32	-		0.37	0.08	-	0.44	0.09
4. Normal tomato plants	Virus protein N isolated Before ultracentrifugation	0.74	0.16				0.01			0.04
5. Tobacco mosaic-diseased Turkish tobacco plants	" Virus protein N isolated Before ultracentrifugation	1.62	0.38	-		0.37	0.08	-	0.45	0.08
6. Tobacco mosaic-diseased Turkish tobacco plants	" Virus protein N isolated Before ultracentrifugation	0.69	0.13	+		0.21	0.06	+	0.55	0.15
		1.20	0.79	+		0.02	0.02		0.04	0.04
	Virus protein N isolated	0.41	0.41			0.33	0.09	+	0.47	0.23
							0.04			0.12

* Preparations 1 and 2, 3 and 4, and 5 and 6 may be regarded as comparable, for each set was grown at approximately the same time and worked up under similar conditions.

† The total nitrogen content of the combined extracts was 0.23 mg. per cc. which, if we disregard the nitrogen contained in the second extract which is known to be very small, is equivalent to 0.59 mg. per cc. when based on the 13 cc. of juice or first extract.

weight from the 20 gm. of root tissue corresponds to 0.13 mg. of protein per cc. of root juice. In calculation of the yield of protein, a factor of 6 was used to convert protein nitrogen figures to protein. The figures for protein nitrogen given in Table I may be converted in a similar manner to protein by multiplication by 6.

The 270 gm. of green leaf tissue obtained from five tomato plants inoculated with virus from these roots were treated in the same way, except that a second extract of the pulp was not made. The yield of virus protein following three ultracentrifugations was 110 mg., or 0.6 mg. of protein per cc. of juice (Preparation 2, Table I). This yield is lower than that customarily obtained, due apparently to the fact that the leaf tissue was infected for only 18 days, a period insufficient to permit the maximum concentration of virus to be reached. For example, as may be seen from the results obtained with another group of tomato plants diseased with aucuba mosaic (Preparation 3, Table I) which were infected for a period of 4 weeks in the spring of 1938, the yield of virus protein was about 1 mg. per cc. of leaf juice. In order to learn something of the distribution of virus protein within the plant, this group of plants was divided into leaves, stems, and small roots of a size comparable to that of the roots grown *in vitro*, and each portion worked up separately. It may be seen that the yield of virus protein from the roots was only about one-quarter, and that from the stems only about one-sixteenth, that obtained from the leaves. However, the yield of virus protein from the roots of tomato plants grown in the greenhouse was somewhat larger than that obtained from the roots grown *in vitro*. It may also be seen from a comparison of the data for Preparations 5 and 6 with that of Preparation 3 in Table I that the yields of virus protein from the corresponding portions of the 4 week-diseased Turkish tobacco plants were larger than the yields in the case of the tomato plants. The differences are much greater than can be accounted for on the basis of the two different strains of virus that were used and are in accord with the earlier findings of Loring and Stanley (6) regarding the relative amounts of virus in Turkish tobacco and in tomato plants.

Practically all of the nitrogen contained in the juice from the roots grown *in vitro* was in a form of non-protein nitrogen associated with inactive material of low molecular weight which did not

sediment on ultracentrifugation. The protein nitrogen content of the root juice was only 0.17 mg. per cc., and practically all of this protein sedimented on ultracentrifugation. Since only 0.02 mg. of virus protein nitrogen was isolated from each cc. of root juice, it appears that much of the protein present in the root juice must have been denatured. However, in the case of the juice from roots of tomato plants grown in the greenhouse, much of the protein was in the form of protein of low molecular weight which remained in the supernatant liquid on ultracentrifugation. The analytical data on the juices of normal and diseased tomato plants (Preparation 3 as compared to No. 4, Table I) show that infection caused an increase in the total nitrogen and protein nitrogen content of the leaf juice and an increase in the protein nitrogen content of the stem and root juices, but had little or no effect on the total nitrogen content of the latter juices. It may be noted that, although the total nitrogen content of the juice from the 4 week-diseased tomato leaves was of the same order of magnitude as that of the juice from the roots grown *in vitro*, over half of the nitrogen in the leaf juice was in the form of protein, and about half of this consisted of inactive protein of low molecular weight which did not sediment on ultracentrifugation.

The virus activity, on a protein basis, of material from roots grown *in vitro* and from leaves was determined by means of the half leaf method on three separate occasions and, as may be seen from the results which are given in Table II, no significant difference was found. Although the virus protein concentration of the roots grown in isolated culture is less than about one-fifth that of leaves grown in a greenhouse, the activity of the isolated virus protein is the same. Since the activity of virus proteins has been found to be one of their most characteristic properties, the fact that the protein from the two quite different sources had the same virus activity is of considerable significance.

When Turkish tobacco plants were inoculated with virus protein from the two sources, the diseases that followed were indistinguishable. The virus proteins from the roots and from leaves were found to have similar solubilities and to crystallize in a similar manner. No difference was noted in the precipitin reaction with antiserum to aucuba mosaic virus protein isolated from other material. The isoelectric point of the virus protein

from the leaves, as determined in the Northrop-Kunitz apparatus, was pH 4.1, that of protein from the roots pH 4.0. Through the cooperation of Dr. Wyckoff, the sedimentation constants of the protein from the two sources were determined. Although both sedimenting boundaries were fairly sharp and there was no indication of the presence of material of low molecular weight, the boundary obtained in the case of protein from the roots was less sharp than that obtained with the protein from the leaf tissue. In both cases the protein sedimented with a boundary character-

TABLE II

Virus Activity of Aucuba Mosaic Virus Protein Isolated from Diseased Tomato Roots Grown in Vitro and from Leaves of Diseased Tomato Plants

Test No.	Test plant	No. of leaves in test	Source of virus protein*	No. of lesions†	$\frac{M.D.}{S.D.}$ ‡
1	<i>Phaseolus vulgaris</i>	95	Roots Leaves	55.9 58.2	0.7
2	<i>Nicotiana glutinosa</i>	79	Roots Leaves	47.6 45.0	1.43
3	" "	40	Roots Leaves	47.3 45.6	1.43

* In every instance virus protein at a concentration of 10^{-5} gm. of protein per cc. in 0.1 M phosphate buffer at pH 7 was used as inoculum.

† The figures represent the average number of lesions per half leaf obtained on inoculation of the designated preparation. A given preparation was administered to the right halves of half of the leaves and to the left halves of the remaining leaves in each test.

‡ The figures represent the findings of a statistical analysis of the results. To show a significant difference between the mean number of lesions in any one experiment, the ratio of the difference of the mean (M.D.) to the standard deviation of the difference (S.D.) should not be less than 2.1.

istic of a single molecular species with s_{20}^0 = about 185. From all these comparisons, it may be concluded that the properties of virus protein isolated by differential centrifugation from aucuba mosaic-diseased tomato roots grown in isolated culture and from aucuba mosaic-diseased tomato plants grown in a greenhouse are either identical or nearly identical, and hence that the protein from the two different sources is probably the same. It should be noted, however, that there must be some interference with the production of virus protein in the excised roots, for the concentra-

tion of virus protein in such roots was slightly less than that in the roots and much less than that in the leafy portions of plants grown under normal conditions in a greenhouse.

The isolation of aucuba mosaic virus protein from diseased tomato roots grown in isolated culture demonstrates that production of virus protein of high molecular weight of the type isolated from diseased leaves is not directly dependent upon the chlorophyll mechanism of a plant. This macromolecular virus protein can be built up in cells whose sole food source consists of simple materials of known constitution and structure. These experiments thus represent an advance in eliminating some of the irrelevant factors which tend to obscure the process of virus multiplication as studied in tissues of more complex metabolism.

The writer wishes to thank Dr. Philip R. White for the roots grown *in vitro* used in the present work and for assistance in the preparation of the manuscript.

SUMMARY

A macromolecular protein was isolated by means of differential centrifugation from the juices of aucuba mosaic-diseased tomato roots grown in isolated culture. The properties of this protein were found to be essentially the same as those of aucuba mosaic virus protein isolated from diseased tomato plants grown under normal conditions in a greenhouse. The concentration reached by aucuba mosaic virus protein in excised roots grown in isolated culture was slightly less than that in roots and less than 20 per cent that in the leaf tissue of greenhouse plants.

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THE ENDOCRINE CONTROL OF LIPID METABOLISM IN THE BIRD*

I. THE EFFECTS OF PREGNANT MARE SERUM UPON THE BLOOD AND LIVER LIPIDS OF THE DOMESTIC FOWL

By C. ENTENMAN, F. W. LORENZ, AND I. L. CHAIKOFF

(From the Division of Physiology of the Medical School, and the Division of Poultry Husbandry of the College of Agriculture, University of California, Berkeley)

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In previous reports from this laboratory (1, 2) it was shown that increased lipid contents of both blood and liver are found in the domestic fowl when laying eggs. In the blood the most pronounced rise occurred in the neutral fat, although significant changes were also observed in phospholipids and cholesterol. In the liver, neutral fat only was affected. Since the rise in blood lipids was first found in the pubescent female containing rapidly growing yolks and at no time, from early age to past maturity, occurred in the male bird, it was concluded that ovarian activity is involved in these lipid changes. No correlation was found between the raised lipid level and the duration or intensity of egg production. Thus, while the onset of ovarian activity provides the stimulus for a rise in the lipid level of the blood, the *degree* of ovarian activity as measured by egg production seems to be in no way related to the *extent* of the blood lipid rise.

In order to throw light on the interrelation between lipid metabolism and ovarian activity, studies have been undertaken on the effects of various hormones on lipid metabolism in the bird. The present investigation records the effects of the gonad-stimu-

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lating principle contained in the serum of pregnant mares upon the lipid metabolism of blood and liver.

EXPERIMENTAL

Immature, single comb white Leghorn birds were used in this investigation. They were fed the Poultry Division stock diet, which has been described elsewhere (2). The methods employed for lipid analyses of blood and liver, as well as the manner in which these tissues were removed from the birds, have also been previously recorded (1, 2).

Asmundson and Wolfe (3), Breneman (4), and Asmundson, Gunns, and Klose (5) reported that precocious sexual development of chicks could be obtained by injection of pregnant mare serum. This material is a good source of gonadotropic hormone (6) and has no measurable amounts of other hormones of the anterior pituitary complex. The serum¹ employed contained 50 rat units (7) of gonadotropic hormone per cc. All administrations were made intramuscularly, the birds receiving 150 rat units six times per week; tissues for lipid analyses were taken 24 hours after the last injection of the hormone. All birds were 40 to 54 days old at the time that injections began. This precedes the minimum age of normal puberty in this breed of fowl by more than 2 months. It has been shown in previous reports (1) that the blood lipids remain within well defined limits in this bird during immaturity.

Results

Blood Changes—The lipid levels of control birds and of injected birds are shown in Tables I and II respectively. The range of age of both groups was identical; it fluctuated between 59 and 77 days, when blood samples were taken. The content of the various lipid constituents in the blood of the control group was well within the range of values previously observed for the immature birds (1). It is known that maturity, which is associated in the female

¹ The material employed in this study was kindly furnished and assayed by the Cutter Laboratories, Berkeley, through the courtesy of Mr. Donald Wonder. It was prepared by defibrinating the serum obtained from mares between the 50th and 120th day of pregnancy. 1 unit of this material represents the minimum amount that will produce corpora lutea 96 hours after the injection into 21 day-old rats.

TABLE I

Blood Lipids of Non-Injected Controls

All lipid values are expressed as mg. per 100 cc. of whole blood.

Bird No.	Age	Weight	Ovary weight	Oviduct weight	Cholesterol			Total fatty acids	Phospho-lipid	Total lipid	Residual fatty acids
					Total	Free	Ester				
	<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>							
02	71	680			112	88	24	300	260	412	108
	77	730	0.25	0.17	139	92	47	242	303	381	105
29	71	820			140	82	58	201	290	341	0
	77	920	0.48	0.15	119	83	36	242	263	361	40
85	71	790			92	80	12	253	236	345	87
	77	870	0.41	0.14	132	92	40	249	273	381	37
10	71	700			107	93	14	234	253	341	55
	77	770	0.38	0.14	135	74	61	198	242	333	0
15	71	640			114	73	41	299	287	413	77
	77	700	0.28	0.18	119	83	36	263	253	382	68
69	69	720			80	70	10	284	231	364	122
	76	840			124	90	34	295	263	419	94
70	76	1010	0.42	0.25	90	74	16	282	308	372	64
55	59	360	0.22	0.06	104	89	15	242	267	346	52

TABLE II

Effect of Daily Injections of 150 Rat Units of Pregnant Mare Serum upon Blood Lipids of Immature Female Birds

All lipid values are expressed as mg. per 100 cc. of whole blood.

Duration of treatment	Bird No.	Age	Bird weight	Ovary weight	Oviduct weight	Cholesterol			Total fatty acids	Phospho-lipid	Total lipid	Residual fatty acids
						Total	Free	Ester				
<i>days</i>		<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>							
8	61	62	650			136	80	56	410	315	546	158
8	62	62	570			133	94	39	494	404	627	194
11	61	65	690	0.55	9.74	106	75	31	385	290	491	168
11	62	65	610	0.47	8.24	101	86	15	426	367	527	169
19	37	64	620	0.43	6.70	128	96	32	424		552	
19	49	64	485	0.49	11.00	191	156	35	1195		1386	
21	22	71	890			223	135	88	1530	727	1753	979
21	16	71	880			157	119	38	959	508	1116	591
21	78	63	710	0.48	11.64	161	107	54	654	540	815	253
21	79	63	650	0.36	5.58	102	84	18	398	449	500	84
22	66	76	760			130	98	32	482	353	612	222
23	88	59	630	0.71	14.19	129	100	29	680	475	809	340
23	512	59	490	0.65	14.00	110	77	33	455	335	565	206
27	22	77	980	1.75	26.92	272	192	80	2220	1040	2492	1465
27	38	77	950	0.89	13.54	180	97	83	598	586	778	145
27	16	77	990	1.11	15.34	189	134	55	1333	636	1522	867
27	81	69	730	0.71	14.55	167	141	26	1050	580	1217	643
27	80	69	750	0.75	18.83	174	149	25	1480	644	1654	1031

with an enormous increase in the lipid content of the blood (1), is not attained in the type of bird studied here until about 120 days. It should therefore be noted that observations were completed on all birds recorded long before the age at which increases in blood lipids could occur spontaneously.

The experimental birds were injected for periods varying between 8 and 27 days. Tremendous increases were observed in the lipid concentrations in the blood, particularly after the longer injection periods. In order to determine whether these increases were statistically significant, the blood samples were divided into

TABLE III

Mean Lipid Values Obtained with Daily Injection of 150 Rat Units of Pregnant Mare Serum for Varying Periods

All lipid values are expressed as mg. per 100 cc. of whole blood.

Lipid constituents	Non-injected	8 to 11 day injection	19 to 23 day injection	27 day injection	<i>F</i> values*
Total cholesterol.....	115	119	148	196	9.91
Free ".....	83	84	108	143	12.90
Ester ".....	32	35	40	53	1.57
Total fatty acids.....	256	429	753	1336	16.19
Phospholipid.....	266	344	484	697	24.29
Total lipid.....	371	548	901	1532	16.18
Residual fatty acids.....	65	173	382	830	12.90
No. of analyses.....	14	4	9	5	

* With the degrees of freedom available an *F* value of 4.64 could be obtained only once in 100 trials, if the value in the different groups were drawn at random from a homogeneous population; consequently higher *F* values are highly significant.

four arbitrary groups. One group contained all blood samples from non-injected birds, while the three other groups contained the results obtained from birds injected for three periods: (1) 8 to 11 days, (2) 19 to 23 days, (3) 27 days. These group values were subjected to an analysis of variance (8). The calculated *F* values are tabulated along with the means for each group in Table III, which shows that the *F* values for all lipid constituents except esterified cholesterol are decidedly greater than 4.64 (the 1 per cent point) and consequently highly significant. Hence the observed differences between the various groups are real and not due

to chance. The individual groups were compared with each other by means of "Student's" t values (8). The mean values for free cholesterol, phospholipid, and neutral fat obtained for birds injected for 19 to 23 days as well as for 27 days were significantly greater than similar values obtained from non-injected birds. As a result of injections over 27 days esterified cholesterol was also increased above its level in the non-injected animals, although in view of the low F value for this lipid constituent the increase should be interpreted with caution. Significant differences were obtained between injection periods of 19 to 23 days and 27 days, strongly suggesting that once a response has been established continued injection of the hormone increases the blood lipids still more. This is illustrated by the behavior of two birds from which blood samples were taken at both 21 and 27 days. The total blood lipids in Bird 16 increased from 1116 to 1522 mg. per cent, in Bird 22 from 1753 to 2492 mg. per cent.

Liver Lipids—The livers of the injected birds contained normal amounts of all lipid constituents. It is particularly interesting to note that abnormal amounts of fat did not appear in the livers of birds despite the fact that they developed severe lipemias.

DISCUSSION

It is clear from the results that *prolonged* administration of gonadotropic hormone obtained from the serum of pregnant mares produces enormous increases in the lipid concentration of the blood of the immature birds. It is particularly interesting to note that the variations in lipid levels observed in the injected birds were strikingly similar to those observed in normal laying birds. Birds injected for 19 to 27 days showed a range of values for total lipid between 500 and 2492 mg. per cent; laying birds previously reported from these laboratories (1) showed a range of values between 551 and 4719 mg., but 90 per cent of these were between 551 and 2486.

By prolonging the period of injection of pregnant mare serum to 6 weeks, Asmundson, Gunns, and Klose (5) succeeded in obtaining ova as large as 5 mm. in diameter in the ovaries of immature birds. In the present study the injection periods were limited to a maximum of 27 days, and although the ovaries increased in size from 2 to 5 times those of the controls, no yolk

growth had begun in any of the birds at the time they were sacrificed for examination (Table II). Hence it may be concluded that the rise in blood lipid level is not secondary to the withdrawal of fat from the blood by rapidly growing yolks.

Relation of Blood Lipid Response to Response of Oviduct—The results obtained in the present study reveal a striking relation

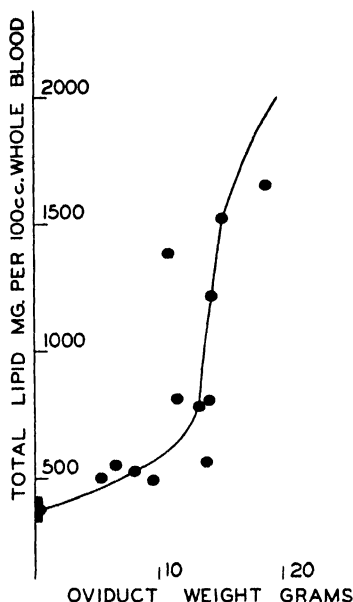


FIG. 1. Relation of blood lipid levels of immature birds to oviduct weight. The symbol at the left end of the curve represents the range of values of non-injected birds; the circles, values from birds injected daily with 150 rat units of pregnant mare serum.

between oviduct size and the level of the blood lipids. This is shown in Fig. 1, in which total fatty acids of the blood are plotted against oviduct weights. This curve suggests that a minimum of ovarian activity sufficient to cause an oviduct growth of at least 10 gm. in birds of the size and age used here must be produced by injections before the blood lipids show a rise.

SUMMARY

1. Continued injections of pregnant mare serum produce a rise in the blood lipids of the immature female bird. These changes are similar to those observed in normal laying birds.

2. It is shown that the formation of yolks is not the stimulus for the rise in blood lipids.

3. A relationship has been demonstrated between the rise in blood lipids and oviduct size. Under the conditions studied a rise in blood lipids occurred when an oviduct growth of at least 10 gm. had been attained by injections.

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STUDIES ON THE PRODUCTION OF TAUROCHOLIC ACID IN THE DOG

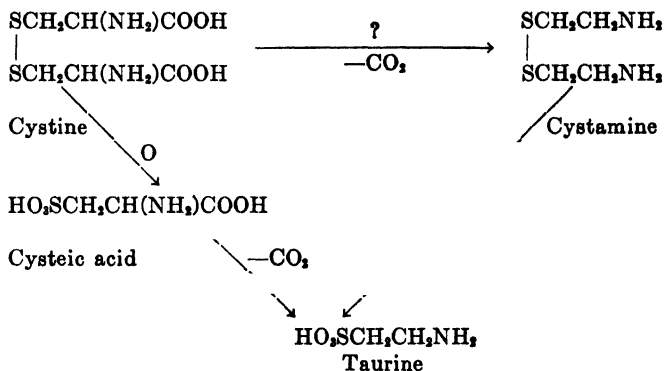
II. CYSTAMINE*

By ROBERT W. VIRTUE AND MILDRED E. DOSTER-VIRTUE

(From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans, and the Department of Chemistry, University of Denver, Denver)

(Received for publication, June 20, 1938)

In recent years there have been several reports (1, 2) concerning the question of utilization of cystamine for growth by the animal organism. It seemed to us that, if cystamine has the power of enabling the animal to grow normally on a low cystine diet, it would probably be able to replace cystine as a precursor of taurine. Robbers (3) has suggested that this may occur. The steps which would be involved in changing cystine or cystamine to taurine are indicated below.



* A preliminary report of these studies was presented before the Thirty-second annual meeting of the American Society of Biological Chemists at Baltimore, April 1, 1938 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. cxxiii (1938)).

Experiments of von Bergmann (4), of Whipple's group (5), and previous results of our own (6) have indicated that the body can produce enough taurine for conjugation with all the cholic acid which is ordinarily available to the dog. Repeated feedings of cholic acid or fasting periods may, however, deplete the body of its supply of taurine. This indicates that cholic acid is the limiting factor in the synthesis of taurocholic acid. We have therefore used fasting bile fistula dogs in an attempt to find whether cystamine may be changed to taurine.

Robbers (3) administered cystamine to dogs and measured their urinary sulfur distribution. Since his dosages were not the same as ours, we have likewise determined the urinary sulfur partition as a measure of the oxidation of the sulfur of the cystamine which was administered.

EXPERIMENTAL

To measure the normal degree of oxidation of the sulfur of cystamine a healthy dog (Dog 38) was catheterized daily, and on the 5th day of a constant diet 0.75 gm. of cystamine dihydrochloride was injected subcutaneously. The animal refused food on that day, thereby invalidating the urinary sulfur figures as a measure of cystamine metabolism. Since cystamine caused a disturbance that prevented the dog from eating normally, the same dog was fasted 3 days and given cystamine dihydrochloride on the 4th day. The urinary sulfur partition was again determined. A bile fistula was later established in this animal, and cystamine was administered once more during a fasting period after recovery from the operation. Bile fistulas were established in all other dogs before the administration of cystamine. All dogs were females.

When the animals had recovered from the operation, they were fasted and then given 6.7 milliequivalents (2.8 gm.) of cholic acid daily by capsule to deplete the liver of its store of taurine. On experimental days 6.7 milliequivalents of cystamine dihydrochloride (0.75 gm.) were either fed or injected subcutaneously at the same time the cholic acid was fed. The bile was collected at 24 hour intervals, just before the capsules were given, and the urines were collected by catheterization.

The analytical methods used have been described previously

(6). To carry out Van Slyke volumetric determinations in Denver it was necessary to extrapolate to obtain weights of moist nitrogen at lower barometric pressures than those given in available sources (7).¹

Results

Table I gives the sulfur partition in the urine of a normal animal (Dog 38, first 5 day period) before and after the administration of cystamine dihydrochloride. The increase in nitrogen excretion on the experimental day may have resulted from a stimulation of general protein catabolism by the cystamine. Consequently part of the extra sulfur found in the urine that day may have been due to breakdown of protein of the dog's tissues. The data for the next 4 days for Dog 38 show a corresponding urinary sulfur partition after a bile fistula had been established in the animal. Both before and after the operation the greater part of the extra sulfur which was excreted had been oxidized by the dog. Small but definite increases in disulfide sulfur appeared on the experimental days. These may have been due to unchanged cystamine, for positive cyanide-nitroprusside tests were obtained on those urines.

The other animals (Dogs 31, 35, and 39) on which urinary figures were obtained gave results in agreement with those of Dog 38. The nitrogen values were somewhat elevated on the day of administration of cystamine, which points to a toxic effect from that substance. Dog 35 vomited on two occasions after the feeding of cystamine. Part of the cystamine may have been absorbed before vomiting occurred. The urine contained unchanged cystamine on those 2 days; hence the sulfur partitions are not recorded. Because of difficulty of retaining cystamine when given orally, subcutaneous injections were given to Dogs 35, 39, and 44. Analysis of the urines of Dogs 31, 35, and 39 showed that after either oral or subcutaneous administration of cystamine most of the extra sulfur had been oxidized, and an increase in disulfide sulfur was observed on experimental days.

Because of a peculiarity of the anatomical structure of Dog 44

¹ A table of these values for pressures ranging from 600 to 692 mm. will be furnished by the authors to those interested.

144 Production of Taurocholic Acid. II

we were unable to catheterize it, and cannot report sulfur distribution in the urine.

The taurocholic acid values reveal no marked rises on experi-

TABLE I

Urinary Sulfur Partitions and Taurocholic Acid Production Following Administration of Cystamine to Fasting Bile Fistula Dogs

Each animal was fed 2.8 gm. = 6.7 milliequivalents of cholic acid daily, except in the first 5 day period of Dog 38.

Dog No.	Day	Weight	Total N	Total S	Sulfate S	Organic S	-S-S-S	Taurocholic acid
		kg.	gm.	mg.	mg.	mg.	mg.	mg.
38	1	12.0	3.78	307	181	126	4	Unoperated
	2		2.96	265	153	112	2	
	3		3.08	258	137	121	4	
	4		5.22	473	288	185	10*	
	5	11.2	4.69	350	194	156	3	
38	1	13.1	9.53	379	226	153	39	4718
	2		7.92	387	258	129	11	2293
	3		6.63	492	324	168	14	1641*
	4	11.8	8.51	436	274	162	6	2895
31	1	13.4	5.64	458	409	49	3	2130
	2		4.69	323	269	54	4	1593
	3		6.34	417	333	84	11	1494*
	4	11.5	4.11	257	216	41	2	1493
35	1	10.2	Urine lost					752
	2		3.19	325	279	46	2	526
	3		3.82	Vomited into urine				725*
	4		3.67	275	229	46	2	572
	5		2.80	Vomited into urine				531*
	6		2.86	175	139	36	1	788
	7	8.6	3.63	266	200	66	16	867†
39	1	11.8	5.68	256	150	106	6	3118
	2		4.62	256	158	98	3	2642
	3		5.32	376	231	145	20	2668†
	4	10.7	4.82	288	130	158	14	2393
44	1	13.6	Unable to catheterize					2756
	2							2382
	3							1823†
	4	12.3						818

* 6.7 milliequivalents of cystamine dihydrochloride (0.75 gm.) were given orally.

† 6.7 milliequivalents of cystamine dihydrochloride (0.75 gm.) were injected subcutaneously.

mental days above other days. In fact, with the exception of Dog 35 a downward trend will be seen from the 1st day of the fasting period through the experimental day. In view of these results, it appears that cystamine failed to give rise to taurine.

DISCUSSION

Sullivan, Hess, and Sebrell (1) reported that cystamine could promote the growth of rats approximately 64 per cent as efficiently as could cystine on a low cystine diet. Others (2) have obtained results which point to an inability of cystamine to replace cystine. That cystine may be changed to taurine by the dog is accepted (8). Robbers (3) feels that deamination to form cystamine may be a normal path of cystine metabolism, and that the cystamine formed may be the precursor of taurine. If cystamine can substitute for cystine, the body might be expected to oxidize cystamine to taurine, which would be conjugated with cholic acid and excreted in the bile as taurocholic acid. The results we have obtained furnish no evidence for a conversion of cystamine to taurine, for the taurocholic acid excretion in the bile of our dogs was not increased after the administration of cystamine.

Robbers measured the distribution of urinary sulfur after injecting cystamine into his dogs. Those experiments differed from ours with regard to the manner and frequency of administration. Robbers injected cystamine subcutaneously as a 10 per cent suspension in olive oil. Since at least 1 gm. of cystamine dihydrochloride was given per day, at least 10 cc. of olive oil were injected each time. The injections were continued daily over 5 or more days, which means that at least 50 cc. of olive oil were injected subcutaneously into each animal. Robbers noted the formation of abscesses at the sites of injection. His high values for total sulfur excretion, some of which were greater than the amount of sulfur administered, may well be explained by breakdown of body tissue caused by the abscesses. Our administrations were made orally or subcutaneously in aqueous solution. The cystamine was administered on only 1 day during an experimental period. No abscesses appeared at the sites of injection. We noted an apathy and loss of appetite, as did Robbers, soon after giving the cystamine.

The outstanding differences between our data and those of Robbers relate to the proportion of extra sulfur which was oxidized by the dogs. Robbers found more organic sulfur than sulfate sulfur following the injection of cystamine, while we observed the opposite distribution. The sulfur of cystamine was readily oxidized to sulfate in our experiments. It appears likely that Robbers' high organic sulfur values may have been due to a combination of increased protein catabolism from the abscesses, and to the continued daily administration of the somewhat toxic cystamine.

SUMMARY

1. Cholic acid was fed to fasting bile fistula dogs for several days to enhance their output of taurocholic acid, and thereby deplete their livers of taurine. When cystamine dihydrochloride was administered orally or subcutaneously with the cholic acid on the 3rd or 4th day, no increase in the output of taurocholic acid was noted. Evidently the dogs did not change the cystamine to taurine.

2. The dogs excreted most of the sulfur of cystamine dihydrochloride in the urine as inorganic sulfate.

The authors wish to acknowledge their indebtedness to Dr. R. W. Whitehead of the University of Colorado Medical School for his cooperation in making available facilities for carrying out these experiments.

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THE DECOMPOSITION OF CYSTINE IN AQUEOUS SOLUTION

By JOSEPH I. ROUTH

(From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor, and the Biochemical Laboratory, State University of Iowa, Iowa City)

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It is well known that treatment with acid and alkaline reagents may alter the physical properties of wool. Recently Crowder and Harris (1) have observed a loss of weight in wool within 15 minutes after treatment with 0.05 N sodium hydroxide at 65° in an atmosphere of nitrogen. A 0.7 per cent loss in weight of wool upon boiling in water for 1 hour has been recorded (2) and further changes have resulted from treatment in an autoclave at 135–150° for 30 minutes (3). It has also been observed¹ that hair on prolonged boiling with water suffered a loss in sulfur and cystine. This was confirmed by the heating of 1 gm. samples of wool in 75 cc. of water under a reflux condenser for periods varying from 8 to 96 hours. The residual wool became progressively more yellow and brittle and contained less sulfur and cystine. The nitrogen content was apparently not affected by this treatment.

The phenomena described above presumably are related to changes in the sulfur and cystine content of the animal fibers. Similar changes in the purified amino acid cystine probably occur. The decomposition of cystine and related compounds by acid and alkaline reagents has been extensively studied by Gortner (4), Clarke (5), Andrews (6), and their collaborators. Sodium hydroxide (1.0 M) decomposed cystine slowly at room temperature with the production of small amounts of cysteine and sulfides (6). It has been shown by Shinohara and Kilpatrick (7) that small amounts of cysteine were formed when cystine was treated with 0.2 M hydrochloric acid in an atmosphere of nitrogen for 7 days at

¹ Lightbody, H. D., and Lewis, H. B., unpublished data.

room temperature. When the temperature was increased to 80°, cysteine formation was observed within 6 hours and a trace of hydrogen sulfide was detected after 24 hours. It is of interest to note that after the treatment of wool with weak sodium hydroxide for 15 minutes, there was a considerable decrease in the sulfur and in the cystine (1).

Anslow and Foster (8), in a study of the effect of the absorption of light energy on cystine in hydrochloric acid solution, postulated that the amino acid was dissociated at the disulfide linkage by light of a wave-length of approximately 2500 Å., with the probable formation of cysteine chloride and cysteine.

This paper presents the results of experiments in which the effects of boiling cystine with distilled water have been studied.

EXPERIMENTAL

The decomposition of cystine was carried out in an atmosphere of either air or nitrogen. The nitrogen was purified by passing the gas through a series of absorption bottles containing alkaline pyrogallol, lead acetate solution, and finally water. The moist gas was led into the reaction flask and over the liquid by means of a short tube that passed through a hollow ground glass plug. A longer tube which extended to the bottom of the liquid also passed through the plug and was used for bubbling gas through the solution and for taking samples. The flask was fitted with a reflux condenser. An outlet tube connected the top of the condenser with a train of absorption bottles that contained cadmium chloride solution (5 to 10 per cent). The reaction flask was heated on an electric heater adjusted to permit maintenance of constant temperature (boiling).

In a typical run, 1.0 gm. of cystine and 1 liter of water were added to the reaction flask, the absorption trains were connected, and nitrogen was bubbled through the solution for 30 minutes, after which a change was made to the alternate inlet and the gas was passed over the liquid. The solution was then heated so that boiling occurred in about 30 minutes. All time relationships were measured from the beginning of the boiling of the reaction mixture. Most of the cystine was in solution after 30 minutes of boiling. Aliquots for analyses were withdrawn immediately at the end of the boiling period. The hydrogen sulfide present in

the sample was removed by passing nitrogen through the hot solution. Hydrochloric acid was then added to the sample to give a final concentration of 0.5 N. The remainder of the solution in the reaction flask, hereafter referred to as the experimental solution, was allowed to cool while nitrogen was bubbled through to drive all of the hydrogen sulfide into the absorption train.

Similar experiments were carried out in an atmosphere of air. A wash bottle containing water was substituted for the train of reagents used in the purification of the nitrogen and the moist air was drawn through the system by means of a suction pump. Passage of both gases through the reaction flask was so regulated that about two bubbles a second entered the system from a tube 8 mm. in diameter.

Cystine and cysteine were determined in the aliquots by the Sullivan method as described in a recent paper (9). Hess (10) has shown that when the amount of cystine in a solution is large in comparison to the cysteine, errors in the determination of cysteine are introduced. During this investigation it was found that cystine alone would produce a color in the cysteine determination. As the concentration of the cystine was increased, the cysteine value of the solution correspondingly increased. In order to overcome this error, controls were run on solutions that contained concentrations of cystine approximately the same as those present in the test samples. The cysteine value of the control was then subtracted from that obtained on the samples and a more accurate measure of the true cysteine value was obtained. As a further check on the cystine and cysteine values, the methods of Shinohara were used (11). In addition to the methods mentioned above, the iodometric method as modified by Lavine (12) was applied to the analysis of the cysteine content of the aliquots.

Cysteine hydrochloride was dried over phosphorus pentoxide and was analyzed by both the Sullivan and Shinohara methods. The color developed in the reaction by cysteine was calculated in terms of cystine, in order to make possible the use of the more stable cystine solutions as standards in subsequent determinations.

For the determination of the hydrogen sulfide formed during the heating, the cadmium sulfide precipitated in the absorption bottles described previously was estimated iodometrically. The total sulfur content of the experimental solution was determined by the

Benedict-Denis method. When the heating period was prolonged to 3 hours, elementary sulfur sublimed in the condenser. This was washed down with alcohol and weighed after the evaporation of the solvent. The material showed the characteristic behavior of elementary sulfur; *i.e.*, solubility in carbon disulfide and a monoclinic crystalline form.

Since many investigators have followed the decomposition of cystine in alkaline reaction by the rate of deamination, it was considered essential to test for the liberation of ammonia during the boiling. In the present series standard acid solutions containing indicators were included in the train of absorption bottles which were connected to the reflux condenser. No evidence of the liberation of ammonia was obtained. The constancy of the nitrogen content of the experimental solution (Kjeldahl) confirmed these findings.

The pH of the distilled water used in the experiments and of the solution at the conclusion of the heating period was determined with a glass electrode. Characteristic crystals of cystine were deposited in the reaction flask after the experimental solution had cooled. The quantity of crystals progressively decreased as the time of boiling was increased.

The data for the time intervals presented in Tables I to III are the averages of at least two experiments in each case.

DISCUSSION

It can be seen from the data in Table I that the relationship between the decomposition of cystine and time of heating is approximately linear. Cystine was present in the solution of every experiment and could be demonstrated before hydrogen sulfide could be detected (Tables I and II). A similar formation of cystine prior to the appearance of hydrogen sulfide was also observed by Shinohara and Kilpatrick (7) when cystine was heated in an acid medium. The deposition of sulfur in the condenser was first observed after 3 hours of boiling. When the periods of heating were greater, the ratio of the amounts of hydrogen sulfide produced to sulfur deposited in the condenser was approximately 2:1 (Table II).

A further study of the partition of sulfur in this decomposition of cystine is presented in Table III. Since it was unnecessary to

apply a correction to the cysteine values as obtained by the Shinohara method, these values were used in the calculations. The complete data for all the experiments may be calculated from Tables I and II. Such calculations for a typical short and a more prolonged period of heating are given in Table III. It is clearly indicated that the decomposition is not a simple one and that a number of side reactions are probable. After periods of heating of 1 hour it was observed that in the experimental solution more

TABLE I

Changes in Composition of Solutions of Pure Cystine after Boiling with Distilled Water

All values are expressed in mg. per liter of solution. The column headings, air and nitrogen, indicate the atmospheres in which the experiments were conducted. The initial solution contained 1000 mg. of cystine in suspension per liter of water.

Time	Sullivan method				Shinohara method				Lavine method	
	Cystine		Cysteine		Cystine		Cysteine		Cysteine	
	N	Air	N	Air	N	Air	N	Air	N	Air
<i>hrs.</i>										
0	1000	1000	0	0	1000	1000	0	0	0	0
0.5	1007	1007	10	11	966	983	10	10	10	10
0.75	999	979	22	20	944	956	18	17	19	16
1	967	963	23	20	937	949	20	21	22	22
3	921	912	42	35	847	858	40	42	39	41
6	808	816	45	37	760	767	42	40	45	41
12	634	688	28	30	611	659	30	31	29	30
24	435	468	25	23	437	451	24	23	23	24
48	178	164	15	14	212	219	14	13	15	15

sulfur was present than could be accounted for as cystine and cysteine. This extra sulfur may have been due in part to the presence of elementary sulfur which was mechanically removed from the reflux condenser. Particles of sulfur were observed in the reaction flask after the longer periods of heating; the values for sulfur deposited in the condenser (Table II) may therefore be considered as minimum. Sulfur present as decomposition products of cystine and cysteine (as sulfenic acid, etc.) may also be included in the extra sulfur fraction. That such acidic products

may well be present was indicated by a progressive decrease in the pH of the solution as the time of heating increased (Table II). Similar changes in the reaction were obtained in an atmos-

TABLE II
Decomposition of Cystine by Boiling Distilled Water

All values except pH are expressed as mg. derived from 1.0 gm. of cystine. The column headings, air and nitrogen, indicate the atmospheres in which the experiments were conducted. The initial solution contained 1000 mg. of cystine in suspension per liter of water.

Time <i>hrs.</i>	Experimental solution				Sulfur						pH change in air	
	Total N		Total S		As H ₂ S		Deposited in condenser		Recovered		Be- fore heat- ing	After heat- ing
	N	Air	N	Air	N	Air	N	Air	N	Air		
0	117	117	267	267	0	0	0	0	267	267		
0.5	115	111	260	265	0	0	0	0	260	265	6.8	6.5
0.75	113	117	263	261	0.2	0	0	0	263	261	6.8	6.6
1	117	115	259	259	0.4	0.3	0	0	259	259	6.8	6.3
3	116	111	253	253	6.3	4.5	0.8	1.0	260	258	6.8	6.0
6	113	113	234	234	16.9	15.1	7.2	8.5	258	258	6.9	5.3
12	112	111	203	208	36.7	33.4	17.6	16.1	257	258	6.5	4.5
24	115	114	153	166	70.4	64.2	38.7	37.6	262	268	7.0	4.3
48	111	116	76	105	105.3	100.0	79.2	58.0	261	263	7.0	4.1

TABLE III
*Recovery of Sulfur after Boiling Cystine with Distilled Water in Two
Typical Experiments*

All results are expressed as mg. of sulfur present. Series A was carried out in an atmosphere of nitrogen, Series B in air.

	Series A, 0.75 hr.	Series B, 24 hrs.
In suspension before heating.....	266.7	266.7
" solution after heating (a).....	263.0	163.7
" " as cystine (b).....	252.0	122.8
" " " cysteine (c).....	4.6	6.5
Extra sulfur in solution (a - b - c).....	6.4	34.4
As hydrogen sulfide (d).....	0.2	63.1
" elementary sulfur (e).....	0.0	40.6
Total recovery (a + d + e).....	263.2	267.4

phere of nitrogen. That the extra sulfur present in the experimental solution could not be accounted for as inorganic sulfates was demonstrated by failure to obtain positive tests for the sulfate in acidified aliquots.

Several mechanisms for the decomposition of cystine by boiling distilled water are suggested by the results obtained in this investigation. Preliminary experiments of a similar nature with cysteine indicate that the decomposition of the two sulfur-containing compounds follows a different course. A discussion of the reactions involved in these processes is deferred until the work upon cysteine is completed.

The author wishes to express his gratitude to Dr. R. L. Garner for his suggestions and criticisms throughout the course of this investigation.

SUMMARY

1. Cystine was decomposed by boiling with distilled water. The formation of cysteine, hydrogen sulfide, and elementary sulfur was demonstrated. The decomposition proceeded at essentially the same rate in air as in nitrogen.

2. From 96.3 to 100.6 per cent of the original sulfur could be accounted for in the various sulfur fractions. Not all of the sulfur present at the end of the experimental period could be accounted for as sulfur of cystine, cysteine, hydrogen sulfide, and elementary sulfur. The formation of decomposition products of an acid nature was suggested by the progressive decrease in the pH of the solutions as the time of heating was increased.

3. The fact that the total nitrogen content of the cystine solution remained unaltered indicated that no ammonia was liberated from the reaction mixture and presumably no deamination had occurred.

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ANEMIA STUDIES WITH DOGS*

BY V. R. POTTER, C. A. ELVEHJEM, AND E. B. HART

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

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The present investigation was undertaken to study further the mechanism by which copper acts as a biocatalyst in the synthesis of hemoglobin and to rationalize, if possible, the results obtained by studies on nutritional anemia in rats (1) with those obtained from studies on hemorrhagic anemia in dogs produced by the technique of Whipple and coworkers (2). Dogs were chosen as the experimental animal for several reasons. In the first place it seemed desirable to demonstrate that the "whole milk" technique for producing nutritional anemia could be extended to include the dog. Secondly, since the existing data on hemorrhagic anemia are almost exclusively concerned with the dog, it was logical to gather data by other techniques on this animal. Lastly, the work of Schultze, Elvehjem, and Hart (3, 4) indicated that attempts to investigate the mechanism of the action of copper might be furthered by chemical determinations on blood. Since most of the constituents which we wished to determine required appreciable amounts of blood, we felt that the dog would suit our purpose admirably because of the facility with which large blood samples could be obtained.

Although nutritional anemia has been produced in a number of species (5), we are not aware of any work in which dogs have been placed on exclusive milk diets with rigorous exclusion of iron and copper. It would seem that the best diet for the study of any factor which is necessary for hemoglobin synthesis would be a diet which contained optimum amounts of all the essential factors except the one in question. Thus far we have found that for the

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study of iron and copper in relation to hemoglobin formation a diet of whole milk alone is unexcelled. We have assumed for the present that milk contains all of the organic factors which are needed for normal hemoglobin formation (see (6, 7)). Obviously the assumption will have to be tested experimentally in the case of the dog, but we have preferred to make a comparison of results obtained on this basis with the results already in the literature before looking for any further organic factors. Fontes and Thivolle (8) and Heubner and Frerichs (9) have studied hemoglobin regeneration after hemorrhage in dogs maintained on a diet of milk, rice, and yeast. We felt that if the rice were displaced by additional milk, the yeast would be unnecessary. Our results seem to bear out this idea, since we have maintained dogs in a state of excellent nutrition for more than a year on an exclusive whole milk diet plus iron and copper additions during part of the period.

EXPERIMENTAL

It was planned to use weanling pups for the production of anemia on a whole milk diet, to cure this anemia with a definite intake of iron and copper, and to study the hemoglobin regeneration in the same dogs after the production of anemia by hemorrhage.

The first group of dogs was a litter of seven mongrel collie shepherds. At approximately 6 weeks of age the pups were taken from their mother and placed in cages of galvanized iron with galvanized 1 inch mesh screen bottoms. Experience in this laboratory has shown that anemia studies with rats can be successfully carried out in galvanized cages. The pups were fed raw whole milk *ad libitum*, and their milk supply was replaced with fresh whole milk twice daily. They received 2 drops of percomorph oil once a week to insure a plentiful supply of vitamins A and D. No water was given. Food intake was recorded daily, and hemoglobin and weight records were taken weekly. The animals were free from internal and external parasites.

After 2 weeks on experiment the hemoglobin levels in all dogs were between 7.0 and 8.0 gm. per 100 cc. of blood. After 7 weeks the levels had dropped to about 6.0 gm. At this time the most anemic dog, No. 2 ♂, at 5.22 gm. of hemoglobin per 100 cc., was

given a daily supplement of 20 mg. of Fe, 1 mg. of Cu, and 1 mg. of Mn. There was an immediate improvement in food intake and rate of growth, and a rapid rise in hemoglobin. After the 1st week the rate of increase in hemoglobin became somewhat slower, and after 7 weeks reached a level of 11.49 gm. per 100 cc. of blood. At this time therapy was discontinued. The dog was in excellent nutritive condition. The hemoglobin increased to 12.05 gm. per 100 cc. of blood in the following week and then began a gradual decline as blood was withdrawn. The other six dogs were maintained on the milk diet during this time, *i.e.* until the 15th week, but showed little further diminution in hemoglobin. Growth continued in these dogs but at a much slower rate than in the case of Dog 2 which was receiving therapy. Two of the dogs finally reached levels of 4.96 and 4.79 gm. of hemoglobin per 100 cc., but the other four remained at about 6.0 gm. Since it appeared unlikely that the anemia would progress much further, the pups were placed on therapy at 15 weeks as follows: Dog 1 ♂, 2 mg. of copper + 2 mg. of manganese per day, with 30 mg. of iron added at 6 weeks; Dog 3 ♀, 30 mg. of iron + 2 mg. of manganese per day; Dog 4 ♀, 30 mg. of iron + 2 mg. of manganese per day; Dog 5 ♀, 15 mg. of iron + 2 mg. of copper + 2 mg. of manganese per day; Dog 6 ♀, 30 mg. of iron + 0.5 mg. of copper + 2 mg. of manganese per day; Dog 7 ♀, 30 mg. of iron + 2 mg. of copper + 2 mg. of manganese per day. The iron was repurified and proved to be free from copper by experiments with rats. On the basis of experience with pigs in this laboratory it was felt that 30 mg. of iron and 2 mg. of copper would represent approximately the smallest dosage that would give an optimum response, while 15 mg. of iron and 0.5 mg. of copper would represent critical levels of therapy. The manganese was added to compensate for the low manganese content of milk. As will be brought out later, the optimum dosage of iron and copper for the cure of nutritional anemia in the dog remains to be determined.

Analytical Work

In an attempt to elucidate the mechanism of the action of copper, samples of blood were collected during the latter part of the development of the anemia and during the course of therapy and analyzed for plasma iron, red cell glutathione, and whole

blood copper, in addition to hemoglobin. The results of the first two determinations are largely of a preliminary nature and the methods and data will not be reported in detail here. However, it seems desirable to summarize the work briefly.

Plasma iron was determined on trichloroacetic acid filtrates, with heating to 90° for 5 minutes during the protein precipitation to get quantitative recovery of the iron.¹ The iron was then determined by the bipyridine method. Moore and coworkers (10, 11) have stressed the importance of plasma iron as transport iron and have shown that low values are obtained in iron deficiency states and during rapid hemoglobin regeneration, while high values are obtained in aplastic anemia and pernicious anemia in relapse. In the present work we found that the plasma iron was greatly reduced in the anemic dogs and in fact was near the limits of the method while the dogs were anemic. Whereas normal levels are greater than 100 micrograms of iron per 100 cc. of plasma, the anemic dogs reached levels as low as 10 micrograms or less. Elvehjem and Sherman (12) have reported data which indicate that in the anemic rat iron fed without copper is stored in the liver and not released for hemoglobin formation until copper is fed. In view of Moore's evidence that plasma iron is transport iron we were curious to learn whether or not iron fed in the absence of copper would appear as plasma iron, particularly since work on the rat indicated that the iron might be held in the liver and not escape into the general circulation. We cannot answer this question at present but can merely report that in all cases the plasma iron remained low until hemoglobin levels reached values of 9 to 11 gm. per 100 cc.; *i.e.*, until the acute anemia was cured. We cannot say that dogs on iron alone would have low plasma iron, since we cannot be sure that the slow rate of hemoglobin formation (apparently due to slight copper reserves) which occurred in our dogs on iron alone was not sufficient to keep the level of plasma iron low.

Schultze and Elvehjem (13) determined the glutathione content of the blood in nutritional anemia in both rats and pigs and found a decrease in the former and an increase in the latter. In the present work the blood was centrifuged and the plasma, white

¹ We are indebted to Dr. Carl V. Moore of Ohio State University for checking our own observations on this point.

cells, and at times a purple layer of cells, were aspirated off, leaving the red cells, which were analyzed without washing by the method of Benedict and Gottschall (14). The glutathione was determined as mg. per 100 cc. of red blood cells, per 100 cc. of whole blood, and per gm. of hemoglobin. The latter figure may be of significance if the regeneration involves glutathione. We found levels of approximately 60 mg. of reduced glutathione per 100 cc. of red cells in the dogs at the time they were shifted to therapy. This figure increased to 80 to 85 at the 4th and 9th days of therapy in the case of Dogs 5, 6, and 7 and showed no significant changes in the others. After the initial rise, the figures approached the original (anemic) value and remained at that level for the duration of the experiment. The significance of the temporary rise is rather obscure, since there was just as much hemoglobin regeneration later in the experiment as there was in the first few days, and also just as great an impetus for regeneration in most cases.

Schultze, Elvehjem, and Hart (4) have shown that in anemia due to copper deficiency the blood copper falls to very low levels and have suggested that there is a critical level of blood copper below which hemoglobin regeneration is impossible. They considered that this critical level must lie somewhere between 10 and 30 micrograms of copper per 100 cc. of blood in the case of pigs, whereas the optimum appeared to be greater than 100 micrograms of copper per 100 cc. Other workers (15, 16) have reported an increase in blood copper in anemia after hemorrhage. In the present work we have continued to use the method of Fischer and Leopoldi (see (3)) for the determination of blood copper. In the case of the anemic dogs, before therapy was begun, the levels ranged from 20 to 40 micrograms per 100 cc. of blood (see Table I). It can be seen from Table I that there was an immediate rise in blood copper in all of the dogs, whether they received copper therapy or not. The dogs which received iron and no copper showed an increase in blood copper which was only temporary and within a week had begun to fall back to the anemic level. This was also true for the dog which was receiving only 0.5 mg. of copper per day.

This observation when compared with findings previously reported for pigs (3) indicates that these dogs had a small reserve

of copper which was capable of being mobilized. The anemia was primarily an iron deficiency as is demonstrated by the fact that Dog 1 which was given copper alone produced almost no hemoglobin, while Dogs 3 and 4, on iron alone, produced appreciable amounts of hemoglobin as will be shown in the next section. The dogs which received 2 mg. of copper per day maintained their blood copper fairly well at levels above 100 micrograms per 100 cc.

TABLE I

Copper Content of Dog Blood during Treatment of Nutritional Anemia

Date	Dog 1	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7
Micrograms Cu per 100 cc. blood						
Oct. 12	40	21	29	26	31	36
" 19		20	13	21	106	31
Therapy						
	2 mg. Cu	30 mg. Fe	30 mg. Fe	15 mg. Fe + 2 mg. Cu	30 mg. Fe + 0.5 mg. Cu	30 mg. Fe + 2 mg. Cu
Oct. 23	145	101	106		156	131
" 28	133	61	88	72	48	123
Nov. 4	156	71	59	104	78	162
" 16	108	42	59	158	49	111
Dec. 1	77	52	14	94	42	204
	30 mg. Fe added					
" 8	60					
" 21	118	125	38	126	118	154
Jan. 12	154	41	85	215	209	155
Feb. 2	165	40	42		95	105

Hemoglobin Regeneration

We were surprised at the slow rate of hemoglobin regeneration which occurred in the dogs, since after 6 weeks of therapy all of the animals were still quite anemic. The blood samples had been taken rather frequently and were usually 25 cc. in volume and upon calculation it was found that they represented as much as 25 per cent of the net hemoglobin production. The samples were taken much less frequently from the 6th to the 12th week of therapy and at the end of this time the hemoglobin had reached levels

of 11 to 13 gm. per 100 cc. Considerable growth had taken place during the 3 month period of therapy and this response together with the fact that the blood samples had been quite large made it seem undesirable to judge hemoglobin regeneration solely on the basis of the increase per unit volume of circulating blood. We decided therefore to calculate the hemoglobin content of the dogs at various times during the experimental period as well as to calculate the amount of hemoglobin which was withdrawn when blood samples were taken. For the first calculation it was necessary to know the blood volume of the dog. This we obtained by calculation, assuming the blood volume to be 8 per cent. Although this procedure is open to criticism, a few simple calculations will show that our data cannot be seriously affected by the magnitude of the fluctuations in blood volume which probably did occur during the course of the experiment. In other words, the figures for total hemoglobin production are probably valid to within 15 per cent. This should be borne in mind when studying the tables. The values for hemoglobin per 100 cc. of blood are, of course, based on direct measurement. We have summarized the data on hemoglobin regeneration in Table II, which also includes the relation between the amount of hemoglobin formed and the amount of iron fed; that is, the per cent of the iron fed which appeared as hemoglobin. The amount of data which entered into Table II is so extensive that it cannot all be included in this paper. However, we have produced and cured hemorrhagic anemia in two of the dogs and the data on these dogs are given in more detailed form in Tables III and IV, which illustrate the method of calculating the absolute increase in hemoglobin as given in Table II, although the figures for hemoglobin removed by bleeding are somewhat condensed.

It is apparent from Table II that the per cent of iron utilized for hemoglobin production was quite low in all cases of nutritional anemia, and with the exception of Dog 1 which produced hemoglobin rapidly when the iron was preceded by a period of copper therapy was probably not significantly different. The reason for the poor iron utilization is not fully apparent as yet; possibly it was due to insufficiency of copper in the dogs other than Dog 1, but if this were true one would expect to find greater differences in dogs receiving 2.0 mg., 0.5 mg., and no copper supplement.

The same line of thought would seem to indicate that the amount of iron was not the limiting factor. This leads to the possibility that all of the dogs were deficient in some factor which is as yet unknown. Finally there is the possibility that an appreciable amount of the iron was being used for the synthesis of organic iron compounds other than hemoglobin. If this were the case, one might expect to find a better utilization of iron for hemoglobin formation in animals which have ceased to grow and have been made anemic by hemorrhage, although such an observation would not prove the argument.

TABLE II

Calculated Amount of Hemoglobin Regeneration on Various Combinations of Iron and Copper

The blood volume was assumed to be 8 per cent (see the text).

Dog No.	Daily therapy (2 mg. Mn to all)	Calculated total Hb production		Fe fed which appeared as Hb	
		0-6 wks.	6-12 wks.	0-6 wks.	6-12 wks.
		gm.	gm.	per cent	per cent
1	2 mg. Cu	6.6			
	30 " Fe added		100.7		27.2
3	30 " "	44.1	58.4	11.9	15.8
5	15 " " + 2 mg. Cu	66.5	21.9	36.0	11.8
6	30 " " + 0.5 " "	50.3	65.7	13.6	17.8
7	30 " " + 2 " "	40.6	43.4	11.0	11.7

Anemia Due to Hemorrhage

The first dog (No. 2) which was made anemic by bleeding was brought down to between 6.0 and 7.0 gm. of hemoglobin by severe bleeding and was allowed to come to weight and hemoglobin equilibrium for several weeks, during which the hemoglobin production was very low (Table III). On March 15, the 36th week of the experiment, the dog was placed on iron therapy for a period of 4 weeks. During this time the hemoglobin production was rather low and the per cent of iron fed which appeared as hemoglobin was very low. The blood copper, which had fallen to 34 micrograms per 100 cc., responded to iron in the manner previously noted in nutritional anemia, but the rise in blood copper was very slight (to 58 micrograms) and began to fall after the first

sampling. At the end of the 4th week the dog was given 4 mg. of copper per day in addition to the iron, and the response was

TABLE III
Hemoglobin Production of Dog 2 during Production and Treatment of Nutritional Anemia and Anemia after Hemorrhage

Date	Time on experiment	Weight	Hb per 100 cc. blood	Calculated total Hb in dog	Blood removed in interval*	Hb removed by bleeding	Calculated total Hb production			Blood Cu per 100 cc.
							Total	Per wk.	Fe fed	
1937	wks.	kg.	gm.	gm.	cc.	gm.	gm.	gm.	per cent	micrograms
July 20	2	3.28	7.05	18.50	6	0.39				
Aug. 24	7	4.88	5.22	20.37	35	3.50	2.26	0.45		
" 24	Placed on therapy; 20 mg. Fe + 1 mg. Cu + 1 mg. Mn per day									
Oct. 12	14	12.48	11.49	114.7	310	32.37	97.8	13.97	29.4	
" 12	Taken off therapy									
1938										
Jan. 11	27	16.81	8.20	110.3	728	53.61	28.0	2.15		
" 11	Severe bleeding begun									
Feb. 2	30	15.09	6.31	76.2	170	11.21	19.5	6.50		62
" 22	33									49
Mar. 15	36	14.27	6.45	73.6	25	1.61	8.6	1.43		34
" 15	Placed on therapy; 30 mg. Fe + 2 mg. Mn per day									
" 22	37	15.83	6.46	81.8	25	1.62	9.8	9.8	15.9	58
Apr. 5	39	16.71	7.65	102.3	25	1.91	22.1	11.05	17.9	53
" 12	40	16.49	7.91	104.3	12	0.95	3.9	3.9	6.3	46
" 12	Shifted to 30 mg. Fe + 2 mg. Mn + 4 mg. Cu per day									
" 19	41	17.61	9.07	127.8	10	1.36	24.5	24.5	39.8	86
" 26	42	18.09	10.52	152.2	10	1.05	25.8	25.8	41.8	100
May 3	43	18.91	11.51	174.1	10	1.15	23.0	23.0	40.1†	158
" 10	44	19.21	13.32	204.7	10	1.33	31.8	31.8	51.6	92
" 17	45	19.35	15.10	233.9	10	1.51	30.5	30.5	49.5	57
" 17	Taken off therapy									
" 24	46	19.18	15.55	238.8	10	1.56	6.4	6.4		129
June 8	48	19.12	15.75	240.9	10	1.58	3.7	1.85		79

* The interval is the period including any given date up to but not including the following date. 160 cc. were the maximum amount withdrawn at one bleeding.

† On 6½ days of therapy.

striking. The hemoglobin production was from 25 to 30 gm. per week (average, 27) and the utilization of the iron fed was

from 40 to 50 per cent. After 5 weeks of therapy the hemoglobin had reached a level of 15.10 gm. per 100 cc. of blood, and therapy was discontinued. Concomitantly with the increased hemoglobin production, the blood copper rose rapidly from a level of 46 to a level of 158 micrograms per 100 cc.

The case of Dog 4 is also quite interesting. This animal had shown considerable hemoglobin regeneration on iron alone, although the iron utilization was very poor and the rate of regeneration was very slow. The rise in blood copper which occurred at that time indicated that the animal was not completely deficient in copper. After 3 months of iron therapy the hemoglobin had reached 12.00 gm. per 100 cc. As soon as therapy was discontinued, the hemoglobin level ceased to rise and remained constant for 6 weeks. At the end of this period the dog had been on a whole milk diet for 33 weeks and was 39 weeks old. During the following week, blood was withdrawn three times in amounts of 65, 85, and 38 cc. The next blood sample was taken 6 weeks later and we were surprised to find that the hemoglobin was at the extremely low level of 3.09 gm. per 100 cc. of blood. Calculation showed that during the 6 week interval the dog had lost 80.68 gm. of hemoglobin which could not be accounted for on the basis of blood withdrawn (see Table IV). All of the other dogs maintained their hemoglobin at approximately the same level at which therapy was discontinued, with a very slight fall in the hemoglobin level, and a slight hemoglobin production. For example, Dog 3, which had been given therapy identical with that of Dog 4 during the treatment of nutritional anemia, ended that treatment at a level of 10.76 gm. of hemoglobin per 100 cc. of blood. 23 weeks later the hemoglobin level had only fallen to 10.45 gm. per 100 cc. in spite of the fact that no therapy had been given during this time. Calculation showed a net hemoglobin production of 1.57 gm. per week, equivalent to 0.8 mg. of iron per day. Dog 4 was fed *ad libitum* for about half an hour morning and night (to prevent spillage) during the period of rapid fall, whereas the other dogs had milk before them at all times. It is difficult to see how this would explain the loss in hemoglobin, however. After two successive low readings Dog 4 was given 30 mg. of iron per day for 5 weeks. During this period the hemoglobin regeneration was very poor and the average weekly pro-

duction was only 10.0 gm. per week, equivalent to 16 per cent of the iron fed. This is similar to the record made by the same dog when the same amount of iron was given during the treatment of the nutritional anemia. The hemoglobin production in the second

TABLE IV

Hemoglobin Production of Dog 4 during Production and Treatment of Nutritional Anemia and Anemia after Hemorrhage

Date	Time on experiment	Weight	Hb per 100 cc. blood	Calculated total Hb in dog	Blood removed in interval*	Hb removed by bleeding	Calculated total Hb production			Blood Cu per 100 cc.
							Total	Per wk.	Fe fed	
	wks.	kg.	gm.	gm.	cc.	gm.	gm.	gm.	per cent	micro-grams
1937										
July 20	2	3.68	7.84	23.08	62	3.38				
Oct. 19	15	9.34	4.96	37.06	125	6.79	17.36	1.33		13
" 19	Placed on therapy; 30 mg. Fe + 2 mg. Mn per day									
Nov. 30	21	12.84	7.01	72.01	50	4.0	41.74	6.96	8.8	14
1938										
Jan. 11	27	14.53	12.00	139.49	60	7.16	71.48	11.90	19.3	
" 11	Taken off therapy									
Feb. 22	33	14.44	11.70	135.16	198	21.01	2.83	0.47		
Apr. 20	41	13.54	3.09	33.47	14	0.43	-80.68	-10.08		32
" 20	Placed on therapy; 30 mg. Fe + 2 mg. Mn per day									
" 27	42	13.01	4.30	44.75	10	0.43	11.71	11.71	19.0	41
May 4	43	12.42	4.85	48.19	10	0.48	3.87	3.87	6.3	31
" 11	44	13.82	4.95	54.73	10	0.50	7.02	7.02	11.4	
" 18	45	14.50	6.95	80.62	14	0.97	26.39	26.39	42.7	34
" 25	46	14.75	7.14	84.25	10	0.71	0.97	0.97	1.6	30
" 25	Shifted to 30 mg. Fe + 4 mg. Cu per day									
June 1	47	14.86	11.75	139.68	10	1.18	56.14	56.14	91.0	76
" 8	48	15.78	13.65	172.32	10	1.37	33.82	33.82	54.8	81
" 15	49	16.27	14.72	191.60	10	1.47	20.65	20.65	33.5	89
" 15	Taken off therapy									
" 22	50	16.49	15.63	206.19	10	1.56	16.06	16.06		113

* See Table III.

period of iron therapy was, however, very erratic and during 1 week actually rose to 26 gm. After 5 weeks of iron alone the hemoglobin level was only 7.14 gm. per 100 cc. At this time the dog was given 4 mg. of copper per day in addition to the iron and the hemoglobin level rose rapidly to 14.72 gm. per 100 cc. of blood

in the next 3 weeks, and therapy was discontinued. During this period the average weekly production was 36.9 gm. of hemoglobin. It is possible that the high conversion figure of 91.0 per cent obtained during the 1st week of copper therapy is not to be regarded as the true value for the efficiency of the conversion of iron into hemoglobin, since at this time the animal may well have accumulated iron reserves in its liver during the previous period of iron alone, as is the case in rats (12). The fact remains that the addition of copper resulted in a high rate of hemoglobin production after two successive periods of iron without copper had given slow rates of hemoglobin production.

The blood copper values in the case of Dog 4 followed the same pattern as in the case of Dog 2, although the rise in copper when iron was fed was very slight. When copper was fed, the blood copper rose from a level of 30 to a level of 113 micrograms of copper per 100 cc. The blood copper values attained by both dogs were lower than those which might be considered normal values and may indicate that the dogs needed more than 4 mg. of copper per day.

The hemoglobin production of Dogs 2 and 4 on 30 mg. of iron and 4 mg. of copper per day is of about the same magnitude as the highest which we have seen in the literature, and is indirect evidence that dogs which have been made anemic by hemorrhage require only milk plus iron plus copper for optimum hemoglobin regeneration. Our results compare very closely with those of Fontes and Thivolle (8) who fed 50 mg. of Fe, 3.3 mg. of Cu, and 6.6 mg. of Mn all in the form of globinates, plus 80 mg. of globin, 12.5 mg. of tryptophane, and 25 mg. of histidine per day to a dog maintained on a diet of milk, rice, and yeast, and made anemic by hemorrhage. They reported a net hemoglobin production of 22 gm. per week during the period of therapy, as compared with the 27 and 36 gm. per week which we obtained. Fontes and Thivolle reported a weekly production of 18 gm. of hemoglobin on 600 gm. of calf liver per day, using the same dog which was mentioned above. The conditions employed by Fontes and Thivolle and by us are not strictly comparable with those of Whipple, since he maintained his dogs at a constant level of low hemoglobin by continual heavy bleeding, while we produced anemia by hemorrhage but withdrew only sufficient blood for analysis until

the anemia was cured. Whereas the anemia produced by Whipple is a truly hemorrhagic anemia, the anemia which we produced in the second instance in Dogs 2 and 4 was probably essentially a nutritional anemia, since the bleedings were merely a means of bringing the animals to the anemic state, and the equilibration period allowed the animal time to resynthesize blood elements which did not require iron or copper.

DISCUSSION

The data on blood copper which are already in the literature (4), coupled with our own observations, make it seem likely that a rise in blood copper is associated with an increased hematopoietic activity. Sachs (16) has reported several studies in which he was led to this same conclusion. In all of his work there were apparently appreciable stores of both iron and copper; consequently hemorrhage was followed by a period of rapid hemoglobin regeneration, and a rise in blood copper was observed.

In the present work, dogs which were made anemic by simply excluding iron and copper from their diet showed a temporary increase in blood copper when iron alone was fed, and this increase was associated with a measurable amount of hematopoiesis. It is to be noted that the increased blood copper was not associated with anemia or with a low iron content in the blood but rather with the administration of iron; *i.e.*, copper was mobilized only when hematopoiesis was made possible by giving iron to an iron-deficient animal. In the case of Dog 1, which was given copper alone, the blood copper rose to normal levels but began to fall again as the anemia continued. But as soon as iron was given, the copper was mobilized and the blood copper rose from a level of 60 micrograms per 100 cc. to a level of 165 micrograms per 100 cc. Apparently the important point is that an *increase in blood copper will occur whenever copper reserves are available and are needed*. It has already been pointed out that the normal rate of hemoglobin formation is characterized by a definite level of blood copper which is probably in excess of 100 micrograms per 100 cc.

In the light of the theory regarding copper mobilization, the data of Schultz, Elvehjem, and Hart (3, 4) become increasingly significant. They found that in iron and copper deficiency in pigs the blood copper fell to extremely low levels. However,

Pigs 3 and 7 had levels of 98.5 and 79.8 micrograms of copper per 100 cc. of blood after 14 days of iron therapy (see Table V). It was suggested that the pigs in one litter (Pigs 1 to 8) were primarily iron-deficient and that the animals were not completely copper-deficient. This is borne out by the fact that their livers originally contained relatively large amounts of copper. Actually the levels in Pigs 1, 2, and 4 appear to be abnormally high, since litter mates (Pigs 6 and 8) after iron and copper had much less copper in their livers.

TABLE V
Mobilization of Copper in Anemic Pigs

These data are from Schultze, Elvehjem, and Hart (3, 4).

Fig No.	Treatment	Cu per gm. dry liver	Cu per 100 cc. blood
		micrograms	micrograms
1	Died from anemia	74.9	
2	" " "	102.5	
4*	" " "	42.1	
3	25 mg. Fe, 14 days	17.9	98.5
7	25 " " 14 "	10.2	79.8
5	2 " Cu for 7 days + 4 mg. Cu for 7 more days	81.4	206.0
6	25 mg. Fe + 2 mg. Cu, 7 days	26.4	231.0
8	25 " " + 2 " " 9 "	24.1	224.0

* Erroneously called Pig 3 in an earlier paper (3).

If we accept for the present the theory that copper reserves are mobilized into the blood whenever hematopoiesis occurs, it is appropriate to inquire from whence they come. A partial answer to this question is indicated from the data referred to above (Table V). Pigs which died in the anemic state early in the experiment had high levels of copper in their livers. Litter mates placed on iron alone showed blood copper levels which were relatively high and indicated that a source of copper was available and was being mobilized. That the source may have been the liver is indicated by the fact that the copper content of the livers of the animals fed iron was abnormally low. The pig which was fed copper alone showed an increased copper content in both blood

and liver, while the pigs fed iron and copper showed high blood copper and intermediate values for the liver.

The next question is what becomes of the copper after it is mobilized, and the answer to this is still quite obscure. The most likely place is the red bone marrow, as had been suggested by Sarata (17), although the work of Schultze *et al.* does not indicate any significant change in the copper content of the marrow. Another possibility is the spleen, as the work of Hahn and Fairman (18) might indicate. There is also the possibility that the copper does not accumulate in any organ but that there is a rapid turnover followed by a loss of bodily copper. The rise in blood copper which occurred when iron was fed to Dogs 2 and 4 after hemorrhage was so slight that their reserves must have been extremely low. It is apparent that these dogs were both quite depleted of copper, and the copper content of blood is so low that only a small amount of copper could have been accounted for in the drawn blood. It is known that hemorrhage mobilizes copper into the blood stream (16). It will be highly important to ascertain whether or not this copper is lost from the body at an increased rate after it is mobilized into the blood. In dogs which are maintained in a state of severe anemia, the impetus to mobilize copper into the blood must be very great. It remains to be seen whether or not they are efficient in conserving the copper thus mobilized.

The most striking result of the work thus far has been the contrast between the rate of hemoglobin formation in the growing dogs and in two of the same dogs after the period of rapid growth had been passed and anemia had been produced by hemorrhage. It seems unlikely that there was any fundamental difference in the two anemias, since although the second anemia was produced by hemorrhage the bleeding was not as extensive as that used by Whipple. It also seems unlikely that the higher level of copper which was fed in the cure of the anemia due to hemorrhage was responsible for the increased rate, since pups from a second litter were given 20 mg. of iron and 2 mg. of copper which on a per kilo basis was comparable to the 4 mg. of copper fed to Dogs 2 and 4, and their hemoglobin regeneration rate was similar to that first observed. Nor was the increased weight of the dogs sufficient

to account for the difference. The most obvious point is the fact that in the first instance the dogs were younger and were growing, while in the later experiment the dogs were nearing maturity, although they showed considerable weight gains when placed on therapy.

The records of Dogs 2 and 4 seem to indicate quite definitely that the canine species requires copper for the regeneration of hemoglobin. This has been questioned by Hahn and Whipple (19) in a recent publication. Speaking with reference to copper they state, "it has not been demonstrated to be lacking in any condition *except nutritional anemia in rats*, and so cannot be considered in any way as a limiting factor in the anemia of dogs due to blood loss or in secondary anemias of human beings generally." Although the logic of this statement is questionable *per se*, it becomes doubly so in view of the fact that copper deficiencies have been produced experimentally or observed under natural conditions in a number of species including chickens (20), pigs (21), rabbits (22), sheep (23), and cattle (24). These papers have been in the literature for some time. It seems likely that the copper catalysis of hemoglobin synthesis is a fundamental biological phenomenon. Experimental work on the mechanism of the catalysis provides methods of determining whether or not this fact is of practical significance.

Hahn and Whipple further state that, "no investigator has reported any condition of copper deficiency in man or dog." Yet in a review (25) on hemorrhagic anemia it was stated that, "copper exerts but a very moderate influence permitting regeneration of hemoglobin only to slightly less than one-half of the amount manufactured during iron medication of equivalent dosage." Thus results in the Rochester laboratories have shown that the feeding of copper results in a hemoglobin production above the basal level. It is obvious that this response would be limited by the amount of other factors such as iron which might be present in the basal diet. We feel that the addition of an essential dietary factor to an already adequate diet will not result in a response. Conversely the occurrence of a specific response is evidence *per se* that the diet is inadequate in the given factor. Sturgis and Farrar (26) have reported that when they duplicated

the conditions employed by Whipple they found that dogs on the basal diet received from 1.0 to 1.5 mg. of copper per day, while a dog on liver received 7.0 to 8.0 mg. of copper per day. They pointed out that when comparing results with liver with results on the basal diet it would be desirable to equalize the copper and iron intake of the experimental animals, although their work was not continued to that point.

The results with Dogs 2 and 4 indicated that the per cent utilization of iron was greater when copper was fed with the iron. This does not mean that the iron is more available when copper is present than when it is lacking. The terms "per cent availability" and "per cent utilized" should not be confused. When Hahn and Whipple stated that the availability of iron salts falls off rapidly when 10 times the optimum dose is given, it appears to us that they should have referred to the per cent utilization.

We feel that there is still much to be learned in regard to the copper and iron requirements of the anemic dog, and further, that the requirements may differ in different types of anemia. But they can only be properly evaluated if all the other dietary factors are present. If liver contains organic dietary factors which are not present in milk but are needed for the cure of hemorrhagic anemia in dogs, attempts to isolate these organic factors should be carried out with the effect of the iron and copper content of the liver preparations eliminated by the inclusion of adequate amounts of iron and copper in the basal ration. On the other hand, if the effects of iron or copper or both are to be studied, it would seem desirable to use a diet which contains the minimum amount of these elements and the optimum amount of all other dietary factors. We feel that the milk diet which we have employed facilitates both lines of investigation. At present we are leaving the question open as to whether or not milk supplies all factors except iron and copper in the case of the anemic dog. We wish to emphasize that it is entirely possible that dogs with hemorrhagic anemia may require factors which are present in liver but not in milk, and that these factors may not be needed for the cure of nutritional anemia. However, we feel that the principles outlined above will apply in any anemia study. We feel that it is highly desirable to integrate the results of various

techniques which have been used for the study of anemia, since the same fundamental principles must underlie the formation of hemoglobin and red blood cells in all cases.

SUMMARY

1. An attempt has been made to begin the integration of the conclusions drawn from the nutritional anemia studies with rats in our laboratory and the conclusions drawn from hemorrhagic anemia studies with dogs.

2. Growing dogs were placed on milk diets with the rigid exclusion of iron and copper. After anemia had developed the dogs were placed on various combinations of iron and copper, the highest of which was 30 mg. of iron and 2 mg. of copper per day. The rate of hemoglobin regeneration over a period of 3 months was at such a rate that it was felt that either the levels of iron and copper were too low, or that a third and unknown factor was lacking.

3. After the hemoglobin levels had approached normal, therapy was discontinued, and two of the dogs were made anemic by bleeding, while the remainder of the animals was maintained on whole milk alone. The anemic dogs were apparently well depleted of both iron and copper, and were virtually unable to form hemoglobin on the diet of milk alone. The dogs which were not bled appeared able to maintain their hemoglobin levels on an extremely low intake of iron and copper on the milk alone.

4. The dogs which were made anemic by bleeding responded poorly to a daily dose of 30 mg. of iron alone, but when 4 mg. of copper per day were given in addition, the hemoglobin regeneration proceeded very rapidly.

5. It is concluded that the canine species should be included among those which require copper for hemoglobin regeneration, and that the need for copper for hemoglobin synthesis is probably a general biological property.

6. Blood copper determinations showed that an increase in blood copper is associated with accelerated hematopoiesis.

7. Dogs were maintained for over a year in a state of excellent nutrition on a diet of whole milk with additional vitamin A and vitamin D, with iron or copper, or both fed during periods of therapy.

8. The implications of the results are discussed at length.

We are indebted to Mr. Simon Black for the copper analyses reported here. This aspect of the work is given in a Bachelor of Science Thesis prepared by Mr. Black.

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HEMICELLULOSES FROM COTTONSEED HULLS

By ERNEST ANDERSON, JOHN HECHTMAN, AND
MILLARD SEELEY

*(From the Carnegie Institution of Washington, Division of Plant Biology,
Stanford University, California, and the Department of Chemistry, the
University of Arizona, Tucson)*

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In 1931 Anderson and Kinsman (1) found that the hemicellulose from cottonseed hulls contained *d*-glucuronic acid, *d*-xylose,¹ and a material of unknown composition which they called body X. They suggested that the *d*-glucuronic acid and body X occupied extreme positions in the hemicellulose molecule, while the xylan made up the middle portion. They also suggested that the hemicellulose might be combined with cellulose by an ester linkage.

Norman (2) has summarized recent work on the hemicelluloses and their relation to cellulose, lignin, and pectin. He points out ((2) p. 97) that present views of the structure of cellulose are not compatible with the idea of a linkage between structural cellulose chains and other large molecules. He suggests ((2) p. 62) that hemicelluloses are combined with lignin. He also points out ((2) p. 169) that treatment of plant materials containing lignin and pentoses with acids may lead to the formation of dark colored condensation products of lignin and furfural. He correctly suggests ((2) p. 47) that the body X described by Anderson and Kinsman might be such a product.

The hemicellulose from cottonseed hulls is apparently less complex than most such substances. If it could be freed from the dark body, it would be a very suitable representative of this class for study. This fact led to the present investigation.

¹ Anderson and Kinsman reported the presence of *l*-xylose. The naturally occurring form of xylose was formerly called *l*-xylose but is *d*-xylose.

EXPERIMENTAL

Isolation of Hemicelluloses—Slight modifications of the method described by Anderson and Kinsman (1) were used in the isolation of the hemicelluloses. After thorough extraction of the cottonseed hull bran with boiling acetone, ethanol, and water it was extracted twice, each time for 48 hours, with a cold 5 per cent solution of sodium hydroxide. The combined alkaline filtrates were made slightly acid with hydrochloric acid and the water-insoluble Hemicellulose A was centrifuged out. Water-soluble Hemicellulose B was precipitated from solution by addition of ethanol. The two hemicelluloses were washed thoroughly with dilute ethanol. The total yield was approximately 25 per cent. The separation of the two portions of the hemicellulose is not sharp. Each portion is still a mixture whose composition varies with the exact procedure followed.

Chlorination of Hemicelluloses—After analysis the two hemicelluloses were dissolved separately in 30 times their weight of a 5 per cent solution of sodium hydroxide and centrifuged from traces of insoluble material. The solutions were made slightly acid with hydrochloric acid, and chlorine gas was passed into the solution for 30 minutes. Equal volumes of ethanol were added to the solutions and the purified Hemicelluloses A1 and B1 were centrifuged out and washed free of chlorides with dilute ethanol. They were still faintly colored.

A part of the chlorinated X body was precipitated from the ethanol solution by addition of water. When heated with 12 per cent hydrochloric acid, it gave some carbon dioxide but only a trace of furfural. This body will be investigated later.

Since a single chlorination does not remove all of the X body, the procedure just described was repeated separately on Hemicelluloses A1 and B1. The resulting Hemicelluloses A₂ and B₂ were perfectly white.

Analysis of Hemicelluloses—The uronic acid was determined by the method of Lefèvre and Tollens (3). Xylan was determined by distillation with 12 per cent hydrochloric acid and precipitation of the furfural with phloroglucinol. Specific rotations were made by dissolving the hemicellulose in a 2 per cent solution of sodium hydroxide. The X body was determined by heating the hemi-

cellulose in a bath of boiling water for 17 hours with 25 times its weight of a 4 per cent solution of hydrochloric acid and filtering the insoluble material. The results obtained on analysis of the various hemicelluloses are given in Table I.

An examination of Table I proves that a mixture of hemicelluloses is present. Some of these are soluble in water and must have been combined chemically with some constituent of the cottonseed hulls before treatment with sodium hydroxide. Hexose sugars are absent from the products isolated. On hydrolysis large amounts of *d*-xylose were obtained. No other sugar was found. The hemicelluloses seem to differ from each other merely in the length of the chain of xylan molecules. Numerous analyses of different preparations showed that the composition of the hemi-

TABLE I
Analysis of Hemicelluloses from Cottonseed Hulls

Hemi-cellulose	Xylan	Uronic acid	X body	Total	$[\alpha]_D^{25}$	Xylose per mole uronic acid
	per cent	per cent	per cent	per cent	degrees	mols
A	85.5	7.46	7.3	100.26		16.7
A1	90.0	8.33	2.62	100.95	-88.5	16.0
A2	90.0	7.46	0.00	97.46	-91.2	17.6
B	80.0	10.9	10.5	101.40		10.7
B1	86.0	11.4	1.8	99.20	-75	11.0
B2	86.3	10.64	0.00	96.94	-72	11.8

celluloses obtained by fractional precipitation with ethanol varies with the relative amounts of water and ethanol used. In Hemicelluloses A and B 1 molecule of glucuronic acid corresponds respectively to 189 and 186 gm. of X body. This suggests that the X body is a definite compound and that it is a constituent part of the hemicellulose. Qualitative tests indicated that the X body is not lignin.

Bromination of Hemicelluloses—The X body, even in small amounts, apparently interferes with accurate analysis of the hemicellulose, probably by combining with some of the furfural, as suggested by Norman ((2) p. 169). At the same time analyses of the hemicellulose indicated that several chlorinations caused a slight decrease in the percentages of xylan and uronic acid. Since

bromine is less active than chlorine, its use, instead of chlorine, seemed advisable for removal of the X body.

Hemicelluloses A and B, prepared as already described and chlorinated one time, were dissolved separately in 100 times their weight of a 4 per cent solution of sodium hydroxide. These solutions were filtered until perfectly clear and then made faintly acid with hydrochloric acid, and a slight excess of liquid bromine added. The solutions were allowed to stand at room temperature for 24 hours. As the bromine color slowly disappeared, additional amounts of liquid bromine were added. Hemicelluloses A and B were precipitated from the solutions by addition of alcohol. They were thoroughly washed first by dilute ethanol

TABLE II

Analysis of Hemicelluloses from Cottonseed Hulls, after Treatment with Bromine Water

Hemi-cellulose	Xylan	Uronic acid	Total	$[\alpha]_D^{25}$	Xylose per mole uronic acid
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>degrees</i>	<i>moles</i>
A	92.20	8.50	100.70	-95.86	15.9
A ₁	92.23	8.60	100.83	-91.49	15.6
A ₂	90.70	10.00	100.70	-91.10	13.2
B	87.24	11.77	99.01	-80.48	11.0
B ₁	90.31	12.39	102.70	-81.64	10.1
B ₂	91.15	11.68	102.83	-77.26	11.0

and later with strong ethanol, dried, and analyzed. The analyses of these Hemicelluloses A and B are given in Table II.

Since Hemicelluloses A and B were still slightly colored, they were brominated a second time, as described above. In some cases it was necessary to brominate a third time before all the color disappeared. By solution in sodium hydroxide and acidification Hemicelluloses A and B were next separated into insoluble portions A₁ and B₁ and soluble portions A₂ and B₂. As would be expected Hemicelluloses A₁ and B₂ were relatively large in amounts, while A₂ and B₁ were relatively small in amounts. The materials were perfectly white. Their analyses are given in Table II.

Titration of Hemicelluloses with Standard Alkali—Accurately weighed samples, approximately 0.2 gm., of Hemicelluloses A and

B, which had been brominated, were heated in boiling water for 30 minutes. A few drops of phenolphthalein solution were added and standard sodium hydroxide solution was titrated into the boiling solution until a faint pink color developed and remained for approximately 10 seconds. The per cent carbon dioxide calculated from this titration checked closely that determined by the method of Lefèvre and Tollens.

Longer boiling of the solution caused the disappearance of the original pink color. To give a permanent pink color after boiling for 10 minutes required approximately twice as much standard alkali as required by theory.

SUMMARY

The preparation, from cottonseed hulls, of perfectly white hemi-celluloses is described. The analyses and specific rotations of these substances are given. They are polyuronides of *d*-glucuronic acid and *d*-xylose in which 1 molecule of the acid is combined with from approximately 10 to 16 molecules of the sugar.

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THE KETONIC ESTROGEN OF SOW OVARIES*

BY W. W. WESTERFELD, SIDNEY A. THAYER,
D. W. MACCORQUODALE, AND
EDWARD A. DOISY

*(From the Laboratory of Biological Chemistry, St. Louis University School
of Medicine, St. Louis)*

(Received for publication, August 10, 1938)

The discovery by Aschheim and Zondek (1) of the large amount of estrogenic activity in the urine of pregnant women led most workers to change from ovaries and placenta to the urine of pregnancy as the source of estrogens. Less than 2 years elapsed between the finding of estrogens in urine and the isolation of the first crystalline estrogen, theelin (Doisy, Veler, and Thayer; Butenandt (2)). Since the most active preparations that previously had been obtained from the ovary were less active than theelin, it was assumed by many investigators that the estrogenic activity of the ovary was due to this substance. However, in 1935 MacCorquodale, Thayer, and Doisy (3) isolated dihydrotheelin from the liquor folliculi of sow ovaries. Since over 50 per cent of the activity was obtained in the form of crystalline dihydrotheelin, it seemed that this compound might be the sole estrogen of the ovary. Later, utilizing the reagent developed by Girard and Sandulesco (4) for the separation of ketonic from non-ketonic substances, Westerfeld and Doisy (5) demonstrated the existence of a ketonic estrogen in sow ovaries.

Girard's reagent reacts with the ketones to form derivatives which are soluble in water and insoluble in ether; the non-ketonic estrogens are not altered by this treatment and can be separated from the ketonic derivatives by extraction with ether. After this separation, the ketones can be regenerated by hydrolysis and obtained in the free form.

* The data of this paper were taken from a dissertation presented by W. W. Westerfeld in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

This separation, which was carried out on the phenolic residue obtained by the purification of an extract of sow ovaries, gave 20 rat units of ketonic and 220 rat units of non-ketonic activity per kilo of whole ovaries. The ketonic nature of the "ketone" fraction was confirmed by a second treatment with Girard's reagent and by the inactivation of this fraction upon treatment with semicarbazide.

The estrogen in the ketonic fraction was identified as theelin by the comparison of the behavior of this fraction and of pure theelin when both were examined by a number of characteristic reactions. These tests included (1) reduction of the ketonic fraction both with hydrogen in the presence of a platinum catalyst, and with sodium and alcohol; (2) determination of the partition ratio between 70 per cent alcohol and benzene for the ketonic fraction; (3) determination of the ratio of rat and mouse units for the ketone; (4) determination of the partition ratio of the estrogen after reduction of the ketonic fraction; and, (5) determination of the ratio of rat and mouse units after reduction of the ketone.

The reduction of theelin with a platinum catalyst resulted in the formation of α -dihydrotheelin with an 8-fold increase in activity. This same increase in activity was obtained by a similar reduction of the ketonic fraction from the ovary, while the activities of equilin and equilenin were not appreciably affected by such treatment.

When partitioned between 70 per cent alcohol and benzene, the activity of the ketonic fraction was found to be divided in the ratio of 1:2.5. This is essentially the same ratio given by theelin and equilin, but differs markedly from the 1:6 ratio for equilenin. After reduction of the ovarian ketone, it partitioned in the 1:1 ratio that is typical of dihydrotheelin.

The ovarian ketone gave a ratio of rat unit to mouse unit of 15.5:1, while after reduction this ratio fell to 3.6:1. Both of these values are in good agreement with the respective ratios of 13:1 for theelin and 3:1 for dihydrotheelin. This ratio was found to be 8:1 for equilin and 1:1.5 for equilenin.

The evidence obtained by these methods clearly indicates that theelin is the principal ketonic estrogen in sow ovaries. Both equilin and equilenin were eliminated as possibilities by the results

obtained, and it is exceedingly improbable that any other compound could behave as theelin in each of the five respects described. Similar data were obtained for the ketonic estrogen in human placenta, identifying this estrogen also as theelin. Subsequent to these studies we have separated with Girard's reagent the ketonic fraction from an extract of human placenta, and have succeeded in isolating the major portion of the ketonic activity as pure crystalline theelin. We thus have a direct confirmation of the accuracy and reliability of these methods.

The relative proportions of ketonic and non-ketonic activities in the extract (220 rat units of non-ketonic to 20 rat units of ketonic) may have created a misleading impression of the actual amounts of these two substances present. The identification of the non-ketonic activity with dihydrotheelin and the ketonic activity with theelin has made it possible to calculate the actual weight of each estrogen present. This has been found to be 0.010 mg. of theelin and 0.014 mg. of dihydrotheelin per kilo of whole ovaries. On an actual weight basis the ketonic estrogen constitutes 40 per cent of the total weight of these two estrogens in the ovary.

EXPERIMENTAL

Separation of Ketonic and Non-Ketonic Estrogens

Study of Solutions of Crystalline Theelin and Dihydrotheelin—Studies of mixtures of theelin and dihydrotheelin of known composition have indicated that in the separation by Girard's Reagent T the most likely error is an incomplete recovery of the ketonic estrogen (Table I). The separation is fairly accurate and the ketonic fraction that is obtained is truly ketonic in nature but only 70 to 90 per cent of the total ketones may actually be present in this fraction.

Confirmation of the results for the ketonic fraction was obtained by treating this fraction with semicarbazide. This treatment removes the estrogenic activity of theelin, but has no effect on the activities of such non-ketonic estrogens as dihydrotheelin and theelol.

Separation of Ketonic Estrogen in Sow Ovaries—The separation of ketonic from non-ketonic substances by Girard's reagent was

carried out on two ovarian extracts. The first was an extract of the "residual tissue" of sow ovaries from which the liquor folliculi and corpora lutea had been removed. This tissue was mainly interstitial tissue but also contained all of the follicle walls and many small follicles. The second extract which was studied was prepared from whole ovaries that contained many large follicles. Postmortem changes were minimized in the latter case by obtaining the ovaries from the packing house soon after slaughter; they were hashed with a meat grinder and immediately stirred with 2 volumes of 95 per cent alcohol.

TABLE I
Control of Methods; Girard's Reagent

Substance tested			Non-ketones recovered		Ketones recovered	
Type of solution	Dihydrotheelin	Theelin				
	rat units	rat units	rat units	per cent	rat units	per cent
Crystals in alcohol		870	3	0	865	100
" " "	720		700	97	5	0
Urine extract	300	180	360*	120	130†	72
" "	300	250	350‡	117	210	84
"Unknown" in alcohol	8800	240	8500	97	210	88
" " "	2200	240	1800	82	170	70

* Extract after treatment with semicarbazide, 350 rat units.

† Extract after treatment with semicarbazide, 11 rat units.

‡ Extract after treatment with semicarbazide, 340 rat units.

Both extracts were prepared as follows: The hashed tissue was extracted several times with hot 95 per cent alcohol; the alcohol was distilled and the residue leached with ether. Phospholipids were removed from the ether extract by precipitation with 2 volumes of acetone; the ether-acetone filtrate was distilled and the residue was dissolved in 95 per cent alcohol. Water was added to dilute the concentration of alcohol to 70 per cent, and the cholesterol fraction was removed by extraction with low boiling petroleum ether. The alcohol was distilled from the aqueous phase, and the resulting sludge was extracted with ether. Estrogens were removed from the ether by thorough extraction with 0.5 N NaOH. The alkaline solution was acidified and extracted

with ether, and the acidic substances were washed from the extract with 5 per cent sodium carbonate and water. The phenolic residue obtained by distillation of the ether was dissolved in absolute alcohol and treated with Girard's reagent.

From both extracts a small amount of a ketonic estrogen was separated by this treatment. On a per kilo basis the residual tissue yielded 15 rat units of ketonic estrogen and 300 rat units of non-ketonic estrogen; the values for the whole ovary were, respectively, 20 and 220 rat units. Moreover, this ketonic estrogen was present in an extract of liquor folliculi remaining from our earlier work in which the α -dinaphthoate of dihydrotheelin had been isolated.

A second treatment with Girard's reagent of 360 rat units of the ketonic fraction, previously separated by the same reagent from the extract of whole ovaries, gave a complete recovery of the estrogen in the ketonic fraction. Less than 20 rat units were found in the non-ketonic fraction, the remainder having reacted a second time with Girard's reagent, to be recovered again with the ketones.

The ketonic fractions separated from both ovarian extracts lost their estrogenic activity when treated with semicarbazide. Such treatment reduced the activity of a preparation from 670 mouse units to less than 50 mouse units. This was a true inactivation, undoubtedly due to the formation of the semicarbazone derivative, for the equivalent of 530 mouse units was restored by acidic hydrolysis of an aliquot. When applied to the extracts before fractionation with Girard's reagent, the semicarbazide treatment had no demonstrable effect on the estrogenic activity, since only 5 to 8 per cent of the total rat units was due to the ketonic estrogen.

Identification of Ketonic Estrogen

Only four naturally occurring phenolic and ketonic estrogens have been reported. They are theelin, equilin, equilenin, and hippulin; the existence of the latter substance has not been confirmed. Of these, only theelin seems to be generally distributed in nature. It has been isolated from the urines of pregnant women (2), pregnant mares (6), stallions (7), and men (8), and from

human placenta (9), and palm kernel oil (10). The others have been found only in pregnant mare urine (11). The identity of the ketonic estrogen in sow ovaries was established by comparisons with the crystalline estrogens, theelin, equilin, and equilenin.

Reduction of Ketonic Estrogens; Theelin—One of the outstanding characteristics of theelin is the ease with which it can be reduced to dihydrotheelin. Not only is there a characteristic increase in activity attending this reduction, but the new compound which is formed has distinct characteristics of its own that offer additional means of identification. Thus the identification of the original ketonic material is based on comparisons with two distinct substances, theelin and dihydrotheelin, rather than on just the one substance, theelin.

It was known from the work of Schwenk and Hildebrandt (12) that the reduction of theelin resulted in the formation of two epimers of dihydrotheelin. The epimer with the lower melting point, α -dihydrotheelin, was found to be much more potent in the spayed rat than was the original theelin. The work of others (13) has demonstrated rather conclusively that the reduction of theelin with hydrogen in the presence of a catalyst, or the reduction of theelin with sodium and alcohol results in the formation of principally or entirely the α -dihydrotheelin. Such methods of reduction should therefore enhance the physiological potency of this substance. The relatively inactive β -dihydrotheelin (14) is produced in only small amounts or not at all by these methods.

In this laboratory the reduction of theelin with hydrogen in the presence of a platinum catalyst has been found to give practically a quantitative yield of α -dihydrotheelin. Similar results were reported by Dirscherl (15), who has studied this reduction under varying conditions. He obtained a quantitative yield of α -dihydrotheelin by the catalytic reduction of theelin when the reaction was carried out in alcohol or aqueous alkali. However, when the solvent contained acid, a different type of reaction occurred in which the aromatic ring of the theelin molecule was partially or completely hydrogenated; in addition, the carbonyl group was reduced to a secondary alcohol or to a methylene group. Similar compounds in which the ring was saturated were obtained by Butenandt and by Butenandt, Stormer, and Westphal (16) and found to be physiologically inactive.

It is thus obvious that the reduction of theelin is only attended by an increased physiological activity when the principal product formed is α -dihydrotheelin, and that this is brought about by the catalytic reduction of theelin in neutral or alkaline solutions or by the reduction of theelin with sodium and alcohol.

The actual increase in activity of dihydrotheelin, over that of theelin, has been found to depend not only on the relative proportions of the α and β forms produced, but upon the methods of assay as well. This is particularly true with respect to the use of ovariectomized rats or mice as the test animals. By our procedures the enhancement in activity when theelin is reduced to dihydrotheelin is in the assay with rats 800 per cent, with mice 200 per cent.

Reduction of Other Ketonic Estrogens—The reduction of ketonic estrogens other than theelin has led to less consistent results. David (17) reported that the reduction of equilenin with sodium and alcohol produced an oily product that was more than 3 times as active as equilenin (mouse assay).

By analogy with theelin the increased activity might be due to the formation of a 17-dihydroequilenin. Both epimers of 17-dihydroequilenin were obtained by Marker *et al.* (18) by the reduction of equilenin with aluminum isopropoxide. The lower melting isomer was identified with the 17-dihydroequilenin isolated from pregnant mare urine by Wintersteiner (19), and was found to be about 50 per cent less active than equilenin. The other isomer was found to possess 2 to 3 times the activity of equilenin.¹

The reduction of equilenin in alcohol with the aid of a platinum catalyst was found by Marker (18) to reduce Ring A of the molecule with the loss of the phenolic group. The activity of this substance has not been reported, and it is therefore unknown whether the catalytic reduction of equilenin will enhance or diminish its activity.

David (17) also reported that reduction of equilin with sodium and alcohol gave a dihydroequilin, the activity of which was twice that of equilin in castrated mice. The reduction of equilin

¹ Personal communication from Dr. Kamm. We are also indebted to Dr. Kamm for a sample of equilenin. The equilin was purchased from a chemical supply house.

with a platinum catalyst has not been reported, but the use of a palladium catalyst was attempted by Dirscherl and Hanusch (20). However, instead of hydrogenating the estrogen, this procedure removed hydrogen from the molecule to form equilenin.

In our studies two methods of reduction were used, the catalytic method and the reduction with sodium and alcohol. The former was suggested because of the quantitative yields obtained by this method in the synthetic preparation of α -dihydrotheelin from theelin. It was carried out with the Adams, Voorhees, and Shriner (21) platinum oxide catalyst in a medium of 95 per cent alcohol.

TABLE II
Reduction of Ketonic Estrogens and Ketonic Fraction from Ovary

Substance investigated	Method of reduction	Ketone reduced	After reduction	Enhancement of activity
		rat units	rat units	per cent
Theelin	Sodium and alcohol	250	1000	400
	Platinum catalyst	250	2000	800
Equilin	Sodium and alcohol	85	85	0
	Platinum catalyst	450	600	33
Equilenin	Sodium and alcohol	17	170	1000
	Platinum catalyst	45	90	200
Ketonic fraction from ovary	Sodium and alcohol	25	100	400
		36	300	833
	Platinum catalyst	35	280	800

The reduction with sodium and alcohol was studied because of early failures to obtain results when the catalytic method of reduction was applied to some tissue extracts. The estrogen was dissolved in 10 cc. of absolute ethyl alcohol and 1.5 to 3.5 gm. of sodium were gradually added to the solution in a flask equipped with a reflux condenser. The mixture was kept molten by gentle warming, and additional amounts of alcohol were added as needed. After complete solution of the sodium, water was added, the solution acidified, and the estrogens extracted with ether.

The results obtained by applying these two methods of reduction to each of the three available ketonic estrogens and to the ketonic fraction obtained from the ovary have been summarized in Table II.

Of the three crystalline estrogens only theelin gave appreciable increases in activity by both methods. The results obtained with the platinum catalyst were more convincing, owing to the 8-fold increase in activity which represents a 100 per cent yield of α -dihydrotheelin. Moreover, the increases obtained by the catalytic method were consistent, while the increases obtained by reduction with sodium and alcohol varied from 4-fold to 8-fold.

Neither equilin nor equilenin could be reduced by the platinum catalyst with a marked increase in activity. Moreover, the activity of equilin was not enhanced by the reduction with sodium and alcohol. However, the reduction of equilenin with sodium and alcohol gave a 10-fold increase in activity, exceeding that obtained with theelin by either method.

When applied to the impure ovarian extracts, the reduction with sodium and alcohol never failed to give at least a 4-fold increase in activity, but the catalytic reduction, in some cases, was completely inhibited. This led to the introduction of an additional purification procedure before the ketonic fraction was subjected to the catalytic reduction. The purification was made by a fractional sublimation in a high vacuum, removing nearly all of the solids with the inactive fractions. The active sublimate was recovered, and was then successfully reduced by the catalytic method. The 8-fold increase in activity thus obtained was identical with the results for theelin.

Partition Ratios—Another means used for the identification of the ketonic estrogen in the ovary was a study of the partition ratios between 70 per cent ethyl alcohol and benzene. This ratio was determined by dissolving the estrogen in a known amount of 95 per cent alcohol, adding enough water to give a concentration of 70 per cent alcohol, and extracting this solution once with an equal volume of benzene. The two phases were separated and then assayed to obtain the amounts of estrogen in the two fractions.

A large amount of impurity in a crude extract may alter the solubilities and hence the ratios. Control studies with crude benzene extracts of urine to which the pure estrogens were added have convinced us that these ratios are not influenced by such contaminating substances.

From Table III it can be seen that each of the estrogens studied has a characteristic partition ratio, and that these ratios are in most cases sufficiently distinct from one another to be used as a means of identifying an unknown estrogen.

The partition ratio for the ketonic estrogen of the ovary was found to be 1:2.5. This is satisfactory agreement with the 1:3 ratio for pure theelin but cannot be used to distinguish between equilin and theelin, since both have essentially the same partition ratio. After reduction of the ovarian ketone, the activity partitioned in a ratio of 1:1, which is identical with the ratio for dihydrotheelin.

Ratios of Rat and Mouse Units—A third method used in the identification of the ketonic fraction was a comparison between

TABLE III
Partition Ratios between 70 Per Cent Alcohol and Benzene

Substance investigated	70 per cent alcohol	Benzene	Ratio, alcohol/benzene
	rat units	rat units	
Theelin	350	1050	1:3
Equilin	250	625	1:2.5
Equilenin.	33	200	1:6
Dihydrotheelin	500	500	1:1
Ketonic fraction from ovary.	70	175	1:2.5
“ “ after reduction (aliquot)	25	25	1:1

the number of rat units and mouse units in any one fraction; in this way the relationship between the two units could be established. Examination of the literature revealed that this ratio for theelin was large, while that for dihydrotheelin was relatively small. In Laqueur's laboratory (22) the rat-mouse unit ratio for theelin was found to be 10 to 16:1, and for dihydrotheelin about 6:1. The latter ratio of 5 to 6:1 was also found to be true for equilin by David and de Jongh (23). Schoeller, Dohrn, and Hohlweg (24) have determined the rat-mouse unit ratio to be 5:1 for theelin and 2.5:1 for dihydrotheelin. Other workers have variously determined the rat-mouse unit ratio for theelin to be 5:1 (Rowe and Simond (25)), 10:1 or 36:1 (Hain and Robson (26)), depending upon the methods of assay utilized.

The ketonic fraction which was obtained from the ovarian extract was therefore accurately tested in both rats and mice, and the ratio between the two values gave the ratio of rat and mouse units for the original ketone. A portion of the material that had been catalytically reduced was studied by the same procedure. Both of these figures were then compared with the ratios similarly determined for the crystalline estrogens.

The ratios obtained for the known estrogens and the ovarian fraction are recorded in Table IV. Each of the ratios for the crystalline estrogens is so distinct that minor variations in the accuracy of the assay could not influence the general conclusions. The ovarian ketone had a rat-mouse unit ratio of 15.5:1, while

TABLE IV
Ratio of Rat and Mouse Units

Substance investigated	Amount in preparation		Ratio, rat unit mouse unit
	rat units	mouse units	
Ovarian ketonic fraction (aliquot).....	90	1400	15.5:1
Aliquot of ketonic fraction after reduction..	90	330	3.6:1

Ratios for Crystalline Compounds

Equilin.....	8:1	Theelin.....	13:1
Equilenin.....	1:1.5	Dihydrotheelin.....	3:1

after reduction this ratio fell to 3.6:1. Both of these values are in good agreement with the respective ratios of 13:1 for theelin and 3:1 for dihydrotheelin.

SUMMARY

The following evidence indicates quite clearly that the principal ketonic estrogen of sow ovaries is theelin. Crystalline theelin and the ketonic fraction give similar data in the following tests: (1) inactivation by treatment with semicarbazide, (2) partition ratios between 70 per cent ethyl alcohol and benzene, (3) ratios of the weights of the rat and mouse units, (4) enhancement of activity by reduction of the ketone, (5) partition ratios and rat unit-mouse unit ratios for the reduction product corresponding to dihydrotheelin.

The actual concentrations of theelin and dihydrotheelin per kilo of sow ovaries are 20 rat units of theelin and 220 rat units of dihydrotheelin; this is equivalent to 0.010 mg. of theelin and 0.014 mg. of dihydrotheelin per kilo.

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THE ISOLATION OF THEELIN FROM HUMAN PLACENTA

By W. W. WESTERFELD, D. W. MACCORQUODALE,
SIDNEY A. THAYER, AND EDWARD A. DOISY

(From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis)

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The occurrence of large amounts of estrogenic activity in human placenta has been known since the investigations of Fellner (1). The estrogens in this tissue had been studied from time to time without the attainment of success in the identification of any constituent until Browne (2) succeeded in isolating theelol. The identification of this substance with the theelol obtained from pregnancy urine was made by Butenandt and Browne (3).

In a study of the estrogens in this tissue Collip (4) observed that an estrogenic constituent was soluble in water and insoluble in ether, thus differing in these properties from the known estrogenic substances. This constituent was not identified, but the isolation of theelol from placenta and the later demonstration by Cohen and Marrian (5) that theelol occurs in human pregnancy urine as the glucuronide have given rise to the belief that this unknown water-soluble estrogen in placenta is probably theelol glucuronide. To distinguish between theelol and the water-soluble estrogen in placenta, Collip and coworkers (6) reserved the use of the name "emmenin" for the latter substance.

The occurrence of theelin as the major constituent of the ketonic activity in human placenta was established by the methods previously used for the identification of the ketonic estrogen in sow ovaries. The subsequent isolation of theelin in good yield from placenta confirms the reliability of these methods used in the study of the placental estrogens and in the identification of theelin in sow ovaries.

This study has been concerned only with the identification of the ketonic estrogen, and does not exclude the possibility that

there may be estrogens other than theelin and theelol in this tissue. From 422 kilos of human placenta we obtained 120,000 rat units of non-ketonic and 30,000 rat units of ketonic estrogen. This is a yield of 355 rat units per kilo, of which 20 per cent is ketonic.

Assuming that the 30,000 rat units of ketonic activity was due entirely to theelin, a total of 15 mg. of theelin should have been present in this extract; of this we have isolated in a pure crystalline form 12 mg., a yield of 80 per cent.

EXPERIMENTAL

The identification of theelin in placenta was carried out on an extract of ten human placentas. The phenolic fraction was prepared and separated into ketonic and non-ketonic fractions by Girard's Reagent T. We thus obtained 200 rat units of ketones and 600 rat units of non-ketones per kilo. Confirmation of the ketonic nature of the estrogen separated into the ketonic fraction was obtained through inactivation of this fraction by the semicarbazide treatment.

The identification of the ketonic estrogen as theelin was based on the methods utilized in characterizing the ketonic estrogen in sow ovaries. These included (1) reduction of the ketonic fraction with hydrogen in the presence of a platinum catalyst, or with sodium and alcohol; (2) determination of the partition ratios between 70 per cent alcohol and benzene for the ketone and for its reduction product; and (3) determination of the ratio of rat and mouse units for the ketone and for its reduction product. In every test the ketonic fraction from the placental extract behaved like pure theelin.

35 rat units of the ketonic fraction were successfully reduced by the catalytic method with the 8-fold increase in activity that is typical of theelin. Almost as good results were obtained by the reduction with sodium and alcohol, the preparation increasing in activity from 35 to 225 rat units.

For the ketonic fraction the ratio of rat-mouse units was found to be 420:35 or 12:1, for crystalline theelin 13:1. For the estrogen produced by the reduction of this ketone, the ratio was 800:280 or 2.85:1, for crystalline dihydrotheelin 3:1.

When partitioned between 70 per cent alcohol and benzene,

the activity in the ketonic fraction was divided in a ratio of 1:2.8; *i.e.*, 25 rat units in the alcohol and 70 rat units in the benzene. After reduction, the activity partitioned in a ratio of 1.1:1 (30 rat units in the alcohol to 27 rat units in the benzene). These values are in good agreement with the ratios of 1:3 for theelin and 1:1 for dihydrotheelin.

Isolation of Theelin

Preparation of Extracts of Placenta—The isolation of theelin was carried out on an extract of 702 full term human placentas, the total weight of which was 422 kilos. These placentas were obtained from the delivery room, and were kept frozen until a sufficient number had been collected for convenient processing. They were then hashed, and the pulp was stirred with 2 volumes of 95 per cent alcohol. After standing for several days with occasional stirring, the tissue was filtered off and extracted in a continuous extractor for 24 hours with hot 95 per cent ethyl alcohol.

Both alcoholic extracts were combined, and the alcohol was removed by distillation. The aqueous sludge was then carried to dryness in a continuous still. The dry residue was leached several times with hot 95 per cent alcohol, and the phospholipids were precipitated from this extract by the addition of 2 volumes of acetone. The alcohol-acetone filtrate was distilled, and the resulting residue was redissolved in 95 per cent alcohol. Sufficient water was added to give a concentration of 70 per cent alcohol, and the cholesterol fraction was removed by extraction with low boiling petroleum ether. The alcohol was distilled from the aqueous phase, the resulting sludge diluted with water, and the estrogens extracted with ether.

The phenolic and acidic fractions were removed from this ether extract by thorough washing with 0.5 N NaOH. They were recovered by acidification and extraction with ether, and were then separated by washing out the acids with aqueous sodium carbonate.

The phenolic residue was further purified by dissolving in 50 per cent alcohol acidified with HCl and extracting with low boiling petroleum ether. The major portion of the estrogenic activity

remained in the 50 per cent alcohol, and was recovered therefrom by dilution and extraction with ether.

This residue was then fractionated with Girard's reagent, separating it into ketonic and non-ketonic fractions. In this manner, 30,000 rat units of ketones were separated from 120,000 rat units of non-ketonic estrogens. About twice this amount of estrogenic activity was obtained from human placenta when the extraction was carried out on a much smaller scale; the low recovery in this case was probably due to an incomplete extraction in the initial stage. Two additional treatments of the non-ketonic fraction with Girard's reagent did not appreciably increase the yield of ketones, the second treatment removing about 1000 and the third treatment removing less than 500 rat units to the ketonic fraction.

The residue of ketones was leached with hot 0.1 N NaOH; salt was added to flocculate the colloid and, after chilling, the solution was filtered through a sintered glass filter. The insoluble portion was dissolved in 95 per cent alcohol, and after distillation of the alcohol, the residue was again leached with hot 0.1 N NaOH. Several such leachings dissolved all the estrogens.

The concentration of the alkaline solution was increased to 0.2 N by the addition of strong alkali, and the estrogens were removed from the aqueous phase by repeated extractions with ether. The ether was distilled, and the entire process of leaching and extraction, which effected a substantial purification, was repeated on the residue.

Isolation and Identification of Ketonic Estrogen—The semi-crystalline product obtained by distillation of the final ether extract was dissolved in alcohol, and the theelin converted to the semicarbazone. The solution was evaporated, and the residue washed with water and then recrystallized from 95 per cent alcohol. The semicarbazone was hydrolyzed with 0.5 N HCl in 50 per cent alcohol; upon neutralization of the acid and distillation of the excess alcohol, the free theelin precipitated from solution. This was recrystallized several times from aqueous alcohol by use of a little norit for decolorization. The final product had the identical crystalline form and appearance of the theelin prepared from urine.

The weight of the theelin obtained was 7.0 mg. Simultaneous melting points (uncorrected) taken with standard theelin gave

251.5–253° for the placental theelin and 253–254.5° for the urinary theelin, while an equal mixture of the two melted at 252.5–254°. An equal mixture of equilenin and theelin melted at 222–240°, and an equal mixture of equilin and theelin at 212–235°.

The bioassay indicated identity with crystalline theelin; *i.e.*, an activity of 10,000 international units per mg. For a 1 per cent solution in dioxane $[\alpha]_D^{27} = +163^\circ$.

Microcombustion Analysis—

$C_{18}H_{22}O_2$.	Calculated.	C 79.95, H 8.21
	Found.	“ 80.09, “ 8.30

The semicarbazone had the typical crystalline form of theelin semicarbazone. It was analyzed for nitrogen by the Pregl-Dumas method. Found, N 12.3; calculated for $C_{19}H_{26}O_2N_3 \cdot \frac{1}{2}H_2O$, N 12.49.

The estrogens lost to the petroleum ether in the purification process of partition between 50 per cent alcohol and petroleum ether were recovered, treated with Girard's reagent, and the ketonic fraction was purified as previously described. The theelin obtained upon hydrolysis of the semicarbazone was combined with the filtrates resulting from the recrystallization of the theelin previously isolated, and the entire fraction was treated with α -naphthoyl chloride in the presence of pyridine (7). In this way an additional quantity of 5 mg. of theelin was isolated as the naphthoate. The melting point was 208° (uncorrected) and the melting point of an authentic specimen of theelin α -naphthoate was 210°. The mixed melting point was not depressed.

Microcombustion Analysis—

$C_{28}H_{28}O_3$.	Calculated.	C 82.03, H 6.65
	Found.	“ 81.71, “ 6.88

SUMMARY

The ketonic estrogen of human placenta, was shown by characteristic reactions to be theelin.

Theelin was isolated in crystalline form from placental extracts. The characterization clearly indicates that theelin is the principal ketonic estrogen in human placenta.

We wish to acknowledge the kind cooperation of the Department of Obstetrics, the University Hospitals, and the affiliated Hospitals of St. Louis University in the collection of the placentas used in our work. Moreover, we have been aided by financial assistance from the Theelin Fund administered by the Committee on Grants for Research of St. Louis University.

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THE EFFECT OF MONOiodoacetic ACID ON THE INTESTINAL ABSORPTION OF MONOSACCHA- RIDES AND SODIUM CHLORIDE

BY KALMEN A. KLINGHOFFER*

*(From the Laboratory of Physiological Chemistry, Yale University
School of Medicine, New Haven)*

(Received for publication, August 1, 1938)

Verzar and his coworkers (1) have reported that the injection of monoiodoacetic acid into rats results in a decreased rate of absorption of fats and certain monosaccharides. Cori (2) had previously demonstrated that in normal rats galactose and glucose are absorbed about 3 times as rapidly as xylose; but according to Verzar, following the injection of monoiodoacetic acid the rate of absorption of the first two sugars is reduced to that of xylose, while that of the latter is stated to be unaffected by this poison.

To explain these findings Verzar has suggested that the absorption of galactose and glucose under normal conditions is facilitated by the formation of organic phosphorus compounds, while that of xylose is dependent on diffusion alone. By analogy with its action on muscle metabolism, monoiodoacetic acid is assumed to interfere with phosphorylation processes, and consequently to reduce the absorption rate of glucose and galactose to a level dependent on the processes of diffusion.

The present paper deals with an attempt to confirm Verzar's observations. Gross pathological changes, however, followed the injection into rats of monoiodoacetic acid in doses varying between 6 and 20 mg. per 100 gm. of body weight. These were pyloric spasm,¹ hemorrhagic enteritis, adrenal cortical and medullary hemorrhages, hemoglobinuria, and pharyngeal edema. None of these was consistently found, but most of the animals exhibited

* National Research Council Fellow in Medicine, 1937-38.

¹ Verzar reported a prolonged emptying time of the stomach in experiments with neutral fat.

gastrointestinal lesions, which, in view of the subject under consideration, seemed of particular significance. In addition, variations in the lethal dose were considerable, some animals dying within a few minutes of the injection of as little as 10 mg. per 100 gm., and others dying at intervals up to 8 hours. None of the animals injected with the amounts employed by Verzar and his colleagues, 12 to 16 mg. per 100 gm. of body weight, survived this period.

In consequence of these observations, it was doubted that the action of monoiodoacetic acid was specifically concerned with phosphorylation, and it was suspected that any decreased formation of organic phosphorus compounds might be associated with a decreased absorption of certain sugars, but could not be considered a causative factor. Therefore, in addition to determining the effect of injection of monoiodoacetic acid on the absorption of glucose, its effect on the absorption of xylose and sodium chloride was also studied, there being no evidence to indicate that in the passage of the latter substances through the intestinal wall processes other than those of diffusion are involved.

Methods

Stock female rats of the Yale strain weighing between 120 and 300 gm. were used. They were fasted 24 hours, and then injected subcutaneously with the monoiodoacetic acid.² 30 minutes later glucose, xylose, or sodium chloride solution was given by gavage. 90 to 120 minutes after the gavage the animals were killed by the intraperitoneal injection of nembutal, and the gastrointestinal tract from the esophagus to the descending colon removed, macerated, and extracted with boiling water. The solution obtained was diluted to a convenient volume and analyzed.

For the glucose and xylose estimations a Somogyi (3) zinc filtrate was made, and the subsequent analysis performed by the Benedict (4) method. Chloride was estimated by Hald's (5) modification of Patterson's procedure.

The results are presented in Table I.

As reported by Verzar, the injection of monoiodoacetic acid

² The monoiodoacetic acid used was an Eastman Kodak product. Recrystallized from petroleum ether it had a melting point between 82-84°. It was neutralized with sodium hydroxide before injection.

definitely decreased the rate of absorption of glucose, but in these experiments it did not reduce it to the same level as that of xylose in normal animals.

Contrary to the findings of Verzar, however, the injection of this substance also reduced the rate of absorption of xylose. The difference in the rates of absorption of this sugar in the normal and treated animals is definitely significant, despite considerable variation within each group. The variation may be due in part

TABLE I

Data from Experiments in Which Rats Were Injected Subcutaneously with Moniodoacetic Acid in Doses of 6 to 20 Mg. per 100 Gm. of Body Weight, and Were Subsequently Given by Gavage Glucose, Xylose, or Sodium Chloride Solution*

Solution given	No. of animals	Weight		Monosaccharide or NaCl				Critical coefficient
				Given		Absorbed per hr.		
		Mean	Range	Mean	Range	Mean	Range	
		gm.	gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	
25% glucose								
Normal	7	224	158-300	604	596-605	182 ± 7.5	152-205	7.4
Injected	8	274	150-300	584	550-604	106 ± 7.0	77-127	
7 to 25% xylose								
Normal	12	161	120-240	258	165-378	32 ± 5.0	10- 60	2.6
Injected	12	168	120-240	254	165-386	13 ± 5.0	0- 50	
0.9% NaCl								
Normal	6	177	160-200	45	45	21 ± 1.7†	18- 25†	3.1
Injected	11	168	160-187	45	45	14 ± 1.3†	8- 19†	

* The xylose was estimated as reducing equivalents of glucose.

† Allowance was made for the sodium chloride extracted from gastrointestinal tracts of normal fasted rats in estimating the amounts absorbed.

to the administration of varying amounts of xylose, for when a comparison is made of the percentages of xylose absorbed, the difference is even more marked, the control group absorbing 12.2 ± 1.7 per cent, the poisoned 5.2 ± 1.7 per cent. Furthermore, in every experiment a pair of animals was employed, one normal and one poisoned, and the poisoned animal in every instance had an absorption rate lower than that of the control.

The effect of giving moniodoacetic acid to rats subsequently

given sodium chloride solution by gavage was striking. After 2 hours the normal animals had empty gastrointestinal tracts and distended urinary bladders; the poisoned animals had empty urinary bladders and distended gastrointestinal tracts. Analyses of the gastrointestinal contents established the failure of the latter to absorb a considerable portion of the saline. There was a rough correlation between the dose of monoiodoacetic acid injected and the amount of saline unabsorbed, and two animals receiving only 6 mg. per 100 gm. had almost normal absorption rates.

The defect in absorption cannot be attributed to adrenal damage, as lesions of the adrenal glands were not always found in the animals poisoned with monoiodoacetic acid. Moreover Deuel *et al.* (6) have demonstrated that the glucose absorption of adrenalectomized rats is normal if the animals are maintained in good condition by the administration of sodium chloride. As Verzar has attributed the decreased absorption rate of glucose which he found in the adrenalectomized rat to a similar inability to form organic phosphorus compounds, Deuel's experiments have an additional pertinence to the subject under consideration. As there is no evidence that the absorption of xylose and sodium chloride is in any way associated with phosphorylation processes, and since the intestinal pathology is so severe, it is apparent that the inhibition of glucose absorption by monoiodoacetic acid cannot be due to a specific effect on phosphorylation. A similar result undoubtedly could be secured with other poisons or procedures which so severely damage the gastrointestinal tract, or which cause such early death of the animal.³

SUMMARY

The injection of monoiodoacetic acid causes a decreased absorption of glucose, xylose, and sodium chloride from the intestinal tract of the rat. This decrease is associated with severe intestinal

³ Dogs given monoiodoacetic acid by mouth vomit and may have bloody stools. The effect on the gastrointestinal tract of rats is noted 24 hours after the administration of as little as 3 mg. per 100 gm. This has also been observed by Simon and White (personal communication) who fed the drug to animals on a low protein diet and frequently encountered pyloric hypertrophy and hemorrhagic enteritis.

pathology and is probably caused by it, rather than by any specific action of the drug on intestinal phosphorylation.

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THE ESTIMATION OF ALBUMIN AND GLOBULIN IN BLOOD SERUM

II. SEPARATION OF FRACTIONS BY CENTRIFUGATION WITH THE ANGLE CENTRIFUGE*

BY HOWARD W. ROBINSON, J. WAIDE PRICE, AND
CORINNE G. HOGDEN

(From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati)

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In the clinical methods for the quantitative estimation of serum proteins, filtration, through filter paper, is the usual procedure for the separation of the globulin precipitated from albumin by a 1.50 M sodium sulfate solution. On account of the nature of the precipitate, a highly retentive paper is needed, and also, with most sera, the filtrate must be refiltered many times before it is clear. In a previous communication (1) the authors showed that filter paper adsorbs a definite amount of the soluble protein. Therefore, it is necessary to discard the first portion of the filtrate, because there is a loss of albumin. Later portions are uniform in nitrogen concentration and contain the protein that is soluble in this salt concentration. This protein adsorption was suggested as the cause of the discrepancies in nitrogen values on duplicate albumin filtrates which have been experienced by many workers in employing the micromethod of Howe (2). A detailed procedure for the filtration was outlined in Paper I (1), which, in our hands, has always given like results on duplicate filtrates. Unfortunately, the method requires at least double the amount of serum and, when the quantity of solution that is filtered is increased, the operation becomes very time-consuming. Under these conditions it seemed desirable to search for a substitute or a means of elimination of the filter paper.

* An abstract of this paper was presented before the Thirty-second annual meeting of the American Society of Biological Chemists at Baltimore, March, 1938.

Centrifugation of the globulin precipitate had been suggested as a quick way to obtain a clear albumin solution. Before the development of our present filtering procedure (1), we had attempted to separate the protein constituents in the ordinary centrifuge (International, size No. 1). The serum-sodium sulfate mixtures were placed in 50 cc. Pyrex centrifuge tubes and the eight-place combination head was employed. Centrifugation was carried out in a temperature-controlled room at 38°. The results were very unsatisfactory, as in some cases there was very little sedimentation in a reasonable time and in none were clear supernatant solutions obtained. At the time we were much disturbed by the possibility that the effect of heating of the solution in the centrifuge might influence the solubility of the protein and also cause convection currents that would hinder sedimentation. When the angle centrifuge was brought to our attention, we decided to give it a trial on this problem.

This centrifuge¹ is a small portable instrument in which the centrifuge tubes revolve at an angle of 40° with the vertical. According to the manufacturer, the principle of this machine was discovered by Dr. Ragnar Lundgren, of St. Goran Hospital, Stockholm. He found that the speed of sedimentation is very much greater with the tube in this position. On centrifuging the 1.50 M sodium sulfate-protein mixture for 1 hour, a clear supernatant solution is obtained which, with reasonable care, can be pipetted from the globulin precipitate and the albumin determined on an aliquot. On a series of blood sera, albumin determinations were made on solutions obtained by our filtration procedure with filter paper and on those obtained by centrifugation. The results are shown in Table I. The difficulties caused by filter paper can be avoided and the determinations made in a shorter time and on smaller samples of serum by centrifugation of the globulin precipitate.

Methods

Human, dog, and rabbit sera were used in these experiments. The rabbit blood was obtained from the heart without anesthesia and the dog and human bloods by venous puncture. A solution

¹ The angle centrifuge is imported and sold by Ivan Sorvall, 210 Fifth Avenue, New York.

of 22 per cent sodium sulfate was added to the serum in the proportion of 30 parts to 1 part of serum. For the comparison study, at least 3.5 cc. of serum were treated with 105 cc. of the salt solution. All the precipitations, filtrations, and centrifugations were carried out in a 38° constant temperature room. As a matter of convenience the serum-sodium sulfate mixtures were allowed to stand overnight at 38°, although comparable results could be obtained after standing for 4 hours. 75 cc. of the serum-sodium sulfate mixture were filtered through one sheet of 9 cm. No. 00 Munktell paper in the manner described before ((1) p. 496). Four 5 cc. aliquots for nitrogen determinations were measured from the last 30 cc. of solution that came through the filter. These determinations were made by the micro-Kjeldahl digestion and distillation procedure described before (1).

For centrifugation, 15 cc. portions of the serum-sodium sulfate mixture were placed in the special 15 cc. oval Pyrex centrifuge tubes and the tubes were covered with rubber caps. This tube is a special tube made for the centrifuge which takes full advantage of the principle of the machine, as it reduces to a minimum the distance the precipitated particle travels across the tube. The type SP angle centrifuge head was used with the aluminum² adapters to fit the 15 cc. tubes. If the centrifuge is run at a speed of 4200 R. P. M. for 1 hour in the 38° room, the temperature of the mixture reaches approximately 40°. With longer periods of centrifugation the temperature does not increase above 40°.

So far we have obtained clear supernatant solutions from all mixtures of serum in 1.50 M sodium sulfate solutions after centrifuging for 1 hour. However, there may be some pathological sera, such as those from patients with lipoidal nephrosis, with which it may be impossible to make the separation by centrifugation.

The precipitated globulin is thrown to the bottom and lower end of the outer side of the tube from the axis of rotation. The precipitate does not tend to take a horizontal position, as do red blood corpuscles from plasma, when the tube comes to rest in the

² We lost a number of determinations through breakage of the glass tubes before we learned the desirability of keeping the aluminum adapters in shape by means of a steel form. These adapters are made of very thin material and lose their shape after a few weeks of centrifuging at high speed.

oblique position. The tube is carefully removed from the centrifuge in the inclined position and turned so that the precipitate is on the lower side of the tube. The rubber cap is removed and a 5 cc. Mohr pipette, whose end is attached to a rubber suction tube, is carefully introduced into the clear solution. About 11 cc. of the supernatant can be withdrawn without disturbing the precipitate. 5 cc. aliquots of this solution are measured into 100 cc. Kjeldahl flasks for the nitrogen determinations. In this study all centrifugations were made in two 15 cc. tubes and two samples for analysis were obtained from each supernatant solution.

In most cases the precipitated globulin tends to settle readily in the 1.50 M sodium sulfate solution. At higher salt concentrations the precipitate remains dispersed in the solution or may rise to the top. In a series of experiments to determine solubility curves, we have been able to centrifuge some precipitates from 1.70 M Na_2SO_4 solution and obtain clear supernatants.

For centrifugation of precipitate the 1.50 M solution of sodium sulfate is a much better medium than the 2.025 M potassium phosphate buffer mixture ($\text{K}_2\text{HPO}_4:\text{KH}_2\text{PO}_4 = 2:1$), which has the same salting-out properties (3), because the density of the former solution is not as great as the latter.

Results

The results of a representative group of the determinations are presented in Table I. The rabbits and dogs except Dog D-5 were apparently normal animals that had not been subjected to any other laboratory procedures. Dog D-5 had been on the modified protein-deficient diet of Weech, Goettsch, and Reeves (4) since December 10, 1937. The human subjects were hospital patients; H-1 had a catarrhal jaundice, H-2 subacute bacterial endocarditis, H-3 subacute nephritis of the nephrotic type, and H-4 nephritis. In all our experiments the difference between the two methods has never been greater than 0.07 gm. of protein per 100 cc. of serum. All the values in column (b) in Table I were made from supernatants which were obtained after centrifuging the mixture for 1 hour. This time interval may be longer than that required in most cases, for we have also obtained good results by a half hour of centrifugation. However, a firmer packing of

precipitate is obtained with the longer centrifuging, which makes the withdrawal of clear solution much easier. The results indicate that the use of filter paper may be avoided and determinations of serum albumin made in a shorter time on smaller samples

TABLE I
Serum Albumin Determinations

Comparison of serum albumin concentrations made from solutions when globulin precipitate was removed by (a) filtration at 38° through paper by "correct value" procedure (1); (b) centrifugation at 38° in the angle centrifuge.

Date	Type of serum*	Total serum protein	Non-protein N	Albumin per 100 cc. of serum		Difference (a) - (b)
				Filtered (a)	Centrifuged (b)	
1938		gm. per 100 cc.	mg. per 100 cc.	gm.	gm.	
Jan. 12	R-1	5.95	29	4.62	4.65	-0.03
" 24	R-2	7.24	47	5.28	5.29	-0.01
Feb. 14	R-3	7.72	48	5.26	5.24	+0.02
Jan. 4	D-7	6.00	28	3.81	3.80	+0.01
" 24		5.99	27	4.00	4.00	0.00
" 12	D-8	6.20	28	3.88	3.86	+0.02
Feb. 4		6.89	28	4.28	4.34	-0.06
Jan. 7	D-6	6.53	25	3.83	3.82	+0.01
" 18	D-18A	6.80	32	4.20	4.24	-0.04
" 17	D-5	4.27	20	2.52	2.56	-0.04
Feb. 8		4.17	19	2.22	2.27	-0.05
Jan. 17	D-9	6.52	25	4.05	4.12	-0.07
" 18	H-1	7.63	29	4.37	4.42	-0.05
" 18	H-2	5.95	50	3.63	3.63	0.00
" 19	H-3	4.95	25	2.80	2.86	-0.06
Feb. 7		5.05	25	2.83	2.83	0.00
Jan. 27	H-4	6.24	59	3.45	3.43	+0.02

* R = rabbit, D = dog, H = human.

of serum by employing the angle centrifuge for the separation of the precipitated globulin.

SUMMARY

1. The separation of the precipitated serum globulin from the albumin in 1.50 M sodium sulfate solution can be made by centrifugation with the angle centrifuge.

2. The results of the comparable determinations of serum albumin made by filtration and centrifugation have always been within 0.07 gm. per 100 cc.

3. The advantages of the procedure are, first, that by elimination of the filter paper, the protein adsorption error is avoided and, therefore, smaller amounts of serum may be used for the determination, and, secondly, that centrifugation is much faster than the filtering procedure.

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THE ESTIMATION OF ALBUMIN AND GLOBULIN IN BLOOD SERUM

III. THE PRECIPITATION OF GLOBULIN AT TWENTY-FIVE DEGREES BY SODIUM SULFATE

BY HOWARD W. ROBINSON, J. WAIDE PRICE, AND
CORINNE G. HOGDEN

*(From the Children's Hospital Research Foundation and the Department of
Pediatrics, College of Medicine, University of Cincinnati, Cincinnati)*

(Received for publication, August 1, 1938)

Sodium sulfate is the most widely used precipitating agent in the quantitative estimation of serum albumin and globulin. Its great advantage over ammonium sulfate is that a nitrogen determination may be made directly on the sodium sulfate filtrate, and over sodium sulfite that it is a more stable salt and can be obtained in a higher state of purity. Other precipitating agents, such as magnesium sulfate, zinc sulfate, acetate, and phosphate buffer mixtures, have such high densities at the molar concentrations required that much trouble with bumping and precipitation of salt occurs during the digestion and, besides, the serum-salt mixtures filter more slowly and centrifugation of precipitate is impossible in most cases.

On account of its limited solubility, the opinion has been held that a 1.50 M sodium sulfate solution must be used at a temperature above that of the average laboratory. It has become customary to carry out the filtration procedure at 37° or 38°. Many workers, who either object to the inconvenience and discomfort of this temperature or are not equipped to work at 38°, use the phosphate buffer mixtures with satisfactory results. In our hands the phosphate mixtures were suitable when a macromethod was used, *i.e.* digestions made in 800 cc. Kjeldahl flasks and distillations carried out in the large block-tin condensers, but with the micromethod, *i.e.* digestion in 100 cc. Kjeldahl flasks and the distillations in the steam distillation apparatus of Goebel (1), they

gave us much trouble and we were never able to obtain the close checks on duplicate filtrates that were obtained with sodium sulfate solutions.

At various times, residual quantities of our stock 22 per cent sodium sulfate have stood for many days in the laboratory at room temperature without crystallization of the dissolved salt. Aware of the ease with which sodium sulfate may exist in supersaturated solutions, we never gave much consideration to this observation. One day we tried to obtain crystals from the solution by the introduction of sulfate crystals, and noted that on vigorous agitation the sodium sulfate dissolved. The same technique showed supersaturation when applied to a 30 per cent sodium sulfate solution. This observation led to some rough solubility studies at "room" temperatures. The results indicated

TABLE I
Solubility of Sodium Sulfate Given by Klottmann (2)

Temperature	Concentration of Na ₂ SO ₄	
	moles per l.	gm. per 100 cc.
25	1.846	26.2
20	1.297	18.4
15	0.907	12.9

that the values for the solubility of sodium sulfate given by Klottmann (2) in his extensive solubility studies were correct. His values for the solubility of sodium sulfate at 25°, 20°, and 15° are given in Table I. A 1.5 M solution is saturated at approximately 22.5° and therefore it must be used at temperatures above 22.5°. In many laboratories the average temperature is about 25°. Since working at this temperature has so many advantages, we decided to make comparable determinations of serum albumin in a series of sera, in which the precipitated globulin separation was carried out at 25° and at 38°.

Methods

Stock 22 per cent sodium sulfate solutions were prepared in our constant temperature rooms at 25°±1° and 38°±1°. The nitrogen determinations were made by the methods described in our pre-

vious papers (3-5). Filtration of the serum-sodium sulfate mixtures was made through one sheet of 9 cm. No. 00 Munktell filter paper in the manner described for the avoidance of the protein adsorption error (4), and clear supernatant solutions were also obtained by the use of the angle centrifuge (5), after the serum-sodium sulfate mixtures had stood overnight at the two temperatures. The precipitated protein does not form large aggregates as fast at 25° as it does at 38°, thus slowing the filtration slightly. How-

TABLE II

Serum Albumin Determinations; Precipitation of Globulin with 22 Per Cent Sodium Sulfate Solution at 25° and 38°

Date	Type of serum*	Serum protein	Non-protein N	Albumin				Greatest deviation
				Filtered		Centrifuged		
				38°	25°	38°	25°	
1938		gm. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
Feb. 4	D-8	6.89	28	4.28	4.28	4.34	4.28	0.06
" 8	D-5	4.17	19	2.22	2.23	2.27	2.25	0.05
" 26	D-5	3.70	21	1.97	2.02			0.05
Mar. 3	D-5	3.54	25		2.12		2.12	0.00
Feb. 14	R-4	7.72	48	5.26	5.23	5.24	5.24	0.03
" 18	R-1	6.69	42	5.12	5.16		5.16	0.04
" 18	R-5	7.53	36	5.28			5.26	0.02
" 17	H-5	3.75	30	2.07	2.07		2.08	0.01
Mar. 4	H-6	3.91	42	1.73			1.72	0.01
" 4	H-7	6.86	24	4.99			5.04	0.05

* D = dog, R = rabbit, H = human. Patients H-5 and H-6 were nephrotic and Dog D-5 had been on an experimental protein-low diet since December 10, 1937.

ever, we have never had any trouble in obtaining clear filtrates in a reasonable time, even when the serum-sodium sulfate mixture had stood only 4 hours at 25°.

Results

Table II summarizes a series of albumin determinations made at 25° and 38° on dog, rabbit, and human sera. The results show good agreement at the two temperatures, which indicates that the solubility of the protein must change very little between

the two temperatures. From these experiments we believe that the separation of globulin from albumin in 1.50 M sodium sulfate solution can be safely made at 25° and, therefore, there is no need for working at 38°.

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OPENING OF THE RING OF THE THIOLACTONE OF HOMOCYSTEINE

BY VINCENT DU VIGNEAUD, WILBUR I. PATTERSON, AND
MADISON HUNT

*(From the Department of Biochemistry, School of Medicine,
George Washington University, Washington)*

(Received for publication, August 6, 1938)

A few years ago Baernstein in studying the methionine content of proteins isolated a crystalline sulfur-containing compound which he was able to identify as the hydroiodide of the lactone of homocysteine (1). Apparently the methyl group of methionine was split off as methyl iodide, and the resulting homocysteine was converted to the hydroiodide of the thiolactone by ring closure. The conversion of methionine to this compound was confirmed by Riegel and du Vigneaud (2) who, in addition, made the thiolactone hydroiodide directly from homocysteine. The corresponding hydrochloride was also prepared.

In his earlier work on the compound, Baernstein encountered a very peculiar behavior of the thiolactone when it was treated with dilute alkali. Even though the sulfhydryl group could be quantitatively accounted for by titration after the alkaline treatment, oxidation of the solution did not yield homocystine. In repeating this treatment of the thiolactone, we likewise failed to isolate homocystine. We obtained instead, after oxidation, a high melting, amorphous material which was insoluble in both dilute acid and alkali, and behaved to all intents and purposes like a polymeric compound. Indeed it reminded one of keratin in its insolubility in reagents which did not modify it chemically.

We felt that this rather puzzling behavior warranted further investigation. If the structure assigned to it were correct, the thiolactone should be capable of yielding homocystine upon opening of the ring followed by oxidation.

It occurred to us, however, that when dilute alkali was added to the hydroiodide of the homocysteine thiolactone, 1 molecule of the free basic thiolactone may have been opened by another

before it could be opened by the alkali, thus yielding a homocysteinylhomocysteine thiolactone. Depending on the conditions existing at the moment, this compound might react with itself to give a diketopiperazine, with the alkali to give a dipeptide, or perhaps react with another molecule of the lactone and the resulting compound could in turn react again, etc. Oxidation of such sulfhydryl compounds could therefore yield disulfide compounds of high or low molecular weight as the case might be.

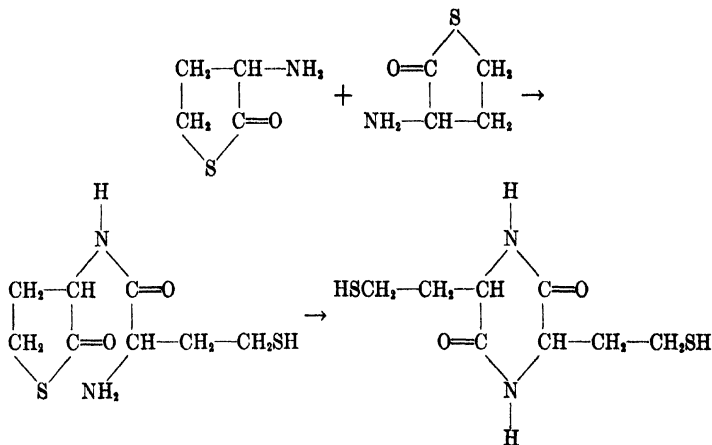
Thinking that the insoluble amorphous material might be some type of polymerized homocysteine, we tried hydrolyzing it with concentrated HCl. An almost theoretical yield of homocysteine was obtained. It was quite clear, therefore, that in the opening of the ring some type of condensation had occurred and that the failure to get homocysteine by alkali treatment and subsequent oxidation was due to this and not to some unorthodox opening which might yield, for example, a γ -hydroxy- α -aminothiol acid.

If such a condensation tends to take place, it should follow that if the thiolactone were opened as quickly as possible by means of strong alkali to avoid the accumulation of any free unopened thiolactone during the reaction, homocysteine should result. Upon oxidation homocysteine should be isolable. We therefore treated the homocysteine thiolactone hydroiodide with 20 moles of 6 N NaOH, and according to prediction, after oxidation with air, an excellent yield of homocysteine was obtained. *dl*-S-Benzylhomocysteine (3, 4) was also obtained by opening the lactone in this manner and treating the solution with benzyl chloride.

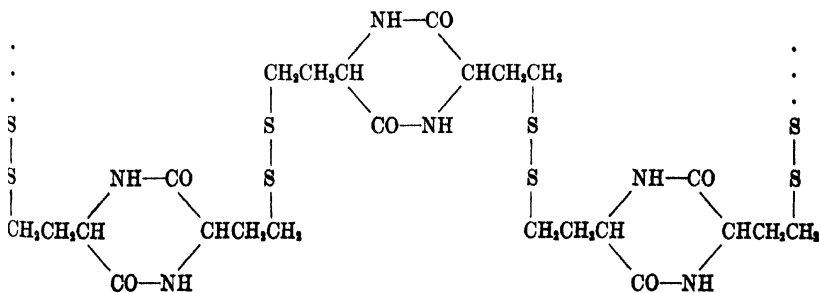
It should also follow from this line of reasoning that if the amino group of the thiolactone were covered by a benzoyl group, treatment of the resulting compound with weak alkali should yield benzoylhomocysteine which upon subsequent oxidation should give dibenzoylhomocysteine. In other words, the type of condensation we have suggested should be prevented. Benzoylhomocysteine thiolactone was therefore prepared, treated with weak alkali, and then aerated. Dibenzoylhomocysteine was obtained in 84 per cent yield and no material similar to that obtained from homocysteine thiolactone was detected.

Having achieved our first objective of finding out whether homocysteine thiolactone could yield homocysteine, we felt it would be of interest to investigate more closely the behavior of

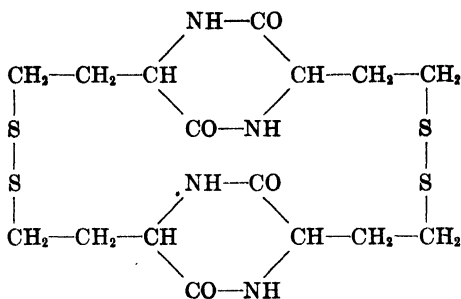
the thiolactone towards dilute alkali to try to establish the nature of the "polymer-like" material and to see if we could obtain evidence as to the type of intermediate involved. On the basis of an analogy to the behavior of α -aminobutyryl- γ -lactone, the formation of the diketopiperazine of homocysteine as an intermediate would seem the most plausible reaction. Fischer and Blumenthal (5) found that when the α -aminobutyryl- γ -lactone, which they were able to isolate from the hydrochloride, was allowed to stand, apparently 1 molecule of this free basic lactone was opened by another and the diketopiperazine of γ -hydroxy- α -aminobutyric acid was formed. In like manner, the diketopiperazine of homocysteine might be formed from homocysteine thiolactone in the following fashion:



Upon oxidation of this sulfhydryl diketopiperazine a disulfide polymer of the following type might result.



On the other hand a dimer analogous to the one prepared by Greenstein (6) in the oxidation of the diketopiperazine of cysteine might be formed as indicated in the following formula.



In investigating the behavior of this lactone hydrochloride with varying strengths of alkali under conditions preventing oxidation, we found that when 1 equivalent of NaOH was added and the solution of the free lactone allowed to stand at room temperature for some hours, a very beautiful crystalline material separated which gave a positive test for the presence of the sulfhydryl group. The composition of this material agreed with that which would be expected for the diketopiperazine. Moreover, acid hydrolysis of the compound yielded homocysteine thiolactone but oxidation did not yield homocystine. This behavior is in accord with what would be expected of such a diketopiperazine. Oxidation of this compound led to the formation of the original insoluble polymer-like compound mentioned above. Apparently this crystalline sulfhydryl compound is an intermediate in the formation of the polymer-like compound. The identity of the former was therefore of much significance, since it would give some indication of the nature of the polymer-like compound. We consequently set about to establish the structure of the crystalline sulfhydryl material.

It is obvious that if this compound were the diketopiperazine of *dl*-homocysteine two inactive modifications should be possible; that is, the racemic and meso forms. The crystalline material was therefore closely investigated for the possibility of its being a mixture. It was found in fact that a fractionation could be

effected by extraction of the material with hot absolute ethyl alcohol. The alcohol-insoluble compound, A, which could be crystallized from pyridine, melted at 237° . It crystallized in elongated hexagons. The alcohol-soluble compound, B, which could be crystallized from absolute ethyl alcohol, melted at 208° . It crystallized in whetstone-like crystals. Both compounds yielded, upon benzylation, derivatives which agreed in composition with that which would be expected for S,S'-dibenzyl derivatives of the diketopiperazine of homocysteine. This coincided with the supposition that Compounds A and B were diastereoisomers of one another, one meso, the other racemic. The benzyl derivative of Compound A melted at 176° and crystallized in clusters of irregular shaped prisms and plates, while that from Compound B possessed a melting point of 165° and crystallized in elongated hexagonal plates.

In order to bring to bear additional evidence for the interpretation of the structure of the two crystalline compounds, A and B, the optically active isomers of homocysteine thiolactone hydrochloride were prepared and their aqueous solutions were treated with 1 equivalent of NaOH in an atmosphere of nitrogen. In both cases a homogeneous crystalline compound was obtained, containing a sulfhydryl group and giving analytical data which agreed with that expected for the diketopiperazine. The optically active compounds melted at 212° and crystallized in needle-like rods, while the benzyl derivatives melted at 183° . The latter crystallized in short, thin prisms. Equal amounts of the active sulfhydryl compounds were then mixed together and recrystallized from absolute alcohol. The recrystallized product was identical in crystalline form with Compound B. It possessed the same melting point, namely 208° , and an equal mixture of the two compounds showed no depression of the melting point. It was therefore clear that Compound B was a racemic isomer. From all indications, Compound A was apparently the meso isomer. These conclusions were borne out by a study of the benzyl derivatives. When the optically active benzyl derivatives prepared by benzylation of the sulfhydryl compound in liquid ammonia were mixed in equal amounts and recrystallized, a compound identical with the benzyl derivative of Compound B was obtained.

In order to place beyond question the suspected diketopiperazine structures of the crystalline compounds obtained by the treatment of the thiolactone hydrochloride with 1 equivalent of alkali, a synthetic approach was undertaken. The diketopiperazine of S-benzyl-*d*-homocysteine was prepared from its methyl ester by heating the free ester at 70° for 16 hours. S-Benzyl-*d*-homocysteine diketopiperazine was isolated from the reaction mixture and crystallized from alcohol. The product melted at 183° and crystallized in short, thin prisms. The benzyl compound obtained from the benzylation of the sulfhydryl compound prepared by the opening of *d*-homocysteine thiolactone hydrochloride melted at 183°. The melting point of a mixture of these two benzyl derivatives was also 183°. Both compounds had the same solubility behavior, the same crystalline form, and the same specific rotation. The molecular weight determined by the Rast method with camphor as a solvent agreed with that calculated for the diketopiperazine of S-benzylhomocysteine. It is therefore clear that Compound B is unquestionably the racemic diketopiperazine of homocysteine and by inference Compound A is the meso isomer. Since the polymer-like material obtained from the oxidation of the optically inactive mixture of the diketopiperazine of homocysteine is apparently the same as that obtained by the treatment of homocysteine thiolactone with alkali in the presence of oxygen, it is clear that this material is most likely a disulfide of the diketopiperazine of homocysteine.

In order to confirm this latter conclusion, we tried to prepare the diketopiperazine of S-benzylhomocysteine from the polymer-like material which was formed from the opening of the *l*-homocysteine thiolactone. It was found that the polymer-like substance could be reduced in liquid ammonia with metallic sodium. Furthermore, benzylation of the reduced material in this medium led to the formation in excellent yield of a benzylthiol derivative. This compound was identical with the synthetic diketopiperazine of S-benzyl-*l*-homocysteine, thus establishing the diketopiperazine nature of the polymer-like material.

Whether the polymer-like compound is a dimer or a polymer, represented by either of the structures given above, presented a difficult question because the insolubility of the substance in all suitable solvents that we investigated precluded a molecular

weight determination. We have been forced to rely for the time being on indirect evidence as to its structure.

It was mentioned earlier that Greenstein had prepared a dimer of the diketopiperazine of cysteine. This compound resulted from the oxidation of cysteine diketopiperazine by H_2O_2 . It will be recalled that our polymer-like material was formed by the oxidation of the sulfhydryl diketopiperazine with atmospheric oxygen. An experiment was therefore carried out in which the diketopiperazine of *l*-homocysteine was oxidized with H_2O_2 according to the directions of Greenstein for the oxidation of the diketopiperazine of cysteine. The product which was obtained in almost theoretical yield was amorphous and had properties similar to those of the polymer-like material referred to previously. Thinking that oxidation in very dilute solution might favor the formation of the dimer, we oxidized 400 mg. of the sulfhydryl diketopiperazine which had been dissolved in 4000 cc. of water. Again the amorphous insoluble polymer-like material was obtained.

A comparison of the properties of the dimer of Greenstein with those of our polymer-like material leads us to suspect that we are not dealing with simply the next higher homologue of Greenstein's compound. There appears to be a more fundamental difference between the two compounds. Greenstein's dimer crystallized readily in long, colorless needles and was soluble in water. Although the exact solubility was not given, it was at least sufficiently soluble in water to obtain a 0.4 per cent solution for the determination of the specific rotation. It was furthermore soluble in glacial acetic acid, so that a molecular weight determination could be made.

Although we cannot decide definitely between the dimer and polymer structures for our amorphous material, we feel that the great insolubility of the compound, its amorphous nature, and its dissimilarity in physical properties to the dimeric disulfide of the cysteine diketopiperazine favor the polymer nature for this disulfide of the diketopiperazine of homocysteine.

EXPERIMENTAL

Preparation of Polymer-Like Substance from dl-Homocysteine Thiolactone Hydrochloride—10 gm. of *dl*-homocysteine thiolactone

hydrochloride and 5.48 gm. of NaHCO_3 were dissolved in 50 cc. of water. 1 drop of FeCl_3 solution was added and air was bubbled through the solution for 36 hours, when the sulfhydryl test was negative. The precipitated amorphous polymer-like compound was washed with dilute HCl to remove any iron, and then washed thoroughly with water, alcohol, and ether. The material decomposed at $260\text{--}270^\circ$. A yield of 6.1 gm. was obtained. The substance was insoluble in all ordinary solvents, hot or cold, including acetic acid, acetic anhydride, formamide, acetamide, phenol, and morpholine. It was barely soluble in liquid ammonia. It dissolved in concentrated H_2SO_4 , but the solution began to darken in a short time, thus indicating that a deep seated decomposition was taking place. The polymer-like material was insoluble in syrupy H_3PO_4 . The following analysis was obtained, corrected for ash and for the loss in weight when the material was dried at 100° *in vacuo*.

Found, C 40.51, H 5.27, N 12.24, S 27.44

Preparation of dl-N-Benzoylhomocysteine Thiolactone—1 gm. of dibenzoylhomocystine (3) in 12 cc. of 70 per cent acetone was treated with 2 gm. of granulated tin and 4 cc. of concentrated HCl . 2 drops of 1 per cent chloroplatinic acid were added and the mixture was refluxed for 2 hours. After the reduction the solution was diluted to 50 cc. with 50 per cent acetone and the tin was precipitated with H_2S . The filtrate from the stannous sulfide was concentrated to dryness *in vacuo* and the residue was extracted repeatedly with boiling benzene. After the benzene extracts had cooled slightly, an oil precipitated, which contained most of the color. This was discarded. The second precipitate, which was deposited later, consisted of large, colorless rod-shaped crystals melting at $134\text{--}136^\circ$.¹ The material gave a negative sulfhydryl test at a pH alkaline to litmus but acid to phenolphthalein. A positive sulfhydryl test was obtained at a pH alkaline to phenolphthalein. Upon hydrolysis with 20 per cent HCl , the homocysteine thiolactone was obtained. The following analytical values were obtained for the *dl-N*-benzoyl thiolactone.

¹ All melting points given are corrected.

$C_{11}H_{11}NO_2S$. Calculated. C 59.71, H 5.01, N 6.33
Found. " 59.81, " 5.14, " 6.62

Conversion of dl-N-Benzoylhomocysteine Thiolactone to Dibenzoylhomocystine—82 mg. of benzoylhomocysteine thiolactone were warmed with 4 cc. of 0.1 N NaOH until solution was complete. The solution, which was still alkaline to phenolphthalein, was aerated until the sulfhydryl test was negative. Acidification with HCl brought about the precipitation of an oil which solidified on standing. This was removed by filtration and the filtrate upon standing deposited clusters of needles which were apparently the same material as the oil first obtained. The combined material weighed 74 mg., which is 84 per cent of the theoretical amount. A sample of dibenzoylhomocystine prepared directly from homocystine gave the same melting point, namely 180–182°, and a mixture of the two compounds showed no depression in melting point.

Preparation of Two Inactive Isomeric Diketopiperazines of Homocysteine from dl-Homocysteine Thiolactone Hydrochloride—20 gm. of dl-homocysteine thiolactone hydrochloride were dissolved in 150 cc. of water and 10.96 gm. of $NaHCO_3$ were added. Nitrogen was bubbled through the solution for 14 hours. The precipitated material was removed by filtration and was washed with water. The yield of crude compound was 14.6 gm. which represents 95.5 per cent of the theoretical yield.

13 gm. of the mixture were extracted with 700 cc. of hot absolute alcohol in three portions. After the alcoholic solution had been cooled, 7.1 gm. of whetstone-like crystals (Compound B) were deposited. The melting point of 208° was unchanged by recrystallization. As shown later, this is the racemic diketopiperazine. The pure compound gave the following analytical data.

$C_6H_{14}N_2O_2S_2$. Calculated. C 41.00, H 6.02, N 11.96
Found. " 40.77, " 6.00, " 11.71

The alcohol-insoluble portion (Compound A) was dissolved in 100 cc. of boiling pyridine with the exception of a small insoluble residue which was discarded. When the pyridine solution was cooled, 2.0 gm. of elongated hexagons which melted at 237° were obtained. The melting point failed to change on recrystallization.

As shown later, this is the meso diketopiperazine. The compound had the following percentage composition.

$C_8H_{14}N_2O_2S_2$.	Calculated.	C 41.00, H 6.02, N 11.96
	Found.	" 40.95, " 5.71, " 11.65

Preparation of Diketopiperazines of S-Benzylhomocysteine from Isomeric Diketopiperazines of Homocysteine—380 mg. of the meso diketopiperazine were suspended in a mixture of 10 cc. of pyridine and 10 cc. of water. 110 mg. of MgO and 0.7 cc. of benzyl chloride were added and the mixture was shaken for 2 hours. 0.2 cc. of benzyl chloride and 36 mg. of MgO were added and the mixture was shaken again for 2 hours. This procedure was repeated until a sulfhydryl test on the solution was negative. A heavy precipitate which had settled out was filtered and was washed with water. The precipitate was then dissolved in 50 cc. of boiling 95 per cent ethyl alcohol. The small amount of insoluble residue was discarded. The filtrate was concentrated to 25 cc. Upon cooling, the solution deposited 210 mg. of the meso diketopiperazine of S-benzylhomocysteine which crystallized in irregularly shaped prisms and melted at 176° . Two recrystallizations from alcohol failed to raise the melting point of this material. The following analytical values were obtained.

$C_{22}H_{26}N_2O_2S_2$.	Calculated, N 6.76; found, N 6.70
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The *dl*-diketopiperazine, which melted at 208° , was benzylated in similar manner. The dibenzyl derivative of this isomer melted at 165° and crystallized in elongated hexagonal plates. The same yield of dibenzyl derivative was obtained in both instances. The following analytical values were obtained.

$C_{22}H_{26}N_2O_2S_2$.	Calculated.	C 63.72, H 6.32, N 6.76, S 15.48
	Found.	" 63.58, " 6.45, " 6.75, " 15.47

Preparation of Optically Active Diketopiperazines from Corresponding Enantiomorphic Homocysteine Thiolactone Hydrochlorides—The active homocysteine thiolactone hydrochlorides were prepared from the corresponding isomers of S-benzylhomocysteine (4) according to the directions given for the *dl* compound (2).

The active isomers melted at 194°. The *l* isomer possessed a specific rotation of $[\alpha]_D^{26} = +21.5^\circ$ for a 1 per cent solution in water, and the enantiomorph had an equal rotation of opposite sign.

10 gm. of *l*-homocysteine thiolactone hydrochloride were dissolved in 100 cc. of water and 5.48 gm. of NaHCO_3 were added. Nitrogen was bubbled through the solution for 24 hours. The precipitate which separated was removed from the solution by filtration. 7.2 gm. of material which melted at 208° were obtained. After one recrystallization from absolute alcohol, the diketopiperazine of *l*-homocysteine melted at 212° and had a specific rotation of $[\alpha]_D^{27} = -53.5^\circ$ for a 1 per cent solution in pyridine. Further recrystallization failed to raise the melting point. The following analytical values were found for the compound.

$\text{C}_8\text{H}_{14}\text{N}_2\text{O}_2\text{S}_2$. Calculated, N 11.96; found, N 11.79

10 gm. of *d*-homocysteine thiolactone hydrochloride were treated just as described above for the *l*-homocysteine derivative. The diketopiperazine was obtained in the same yield and with the same melting point, 212°. It possessed a specific rotation of $[\alpha]_D^{27} = +54.5^\circ$ for a 1 per cent solution in pyridine. The following analytical values were found.

$\text{C}_8\text{H}_{14}\text{N}_2\text{O}_2\text{S}_2$. Calculated, N 11.96; found, N 11.80

Preparation of Dibenzyl Derivatives of Optical Isomers of the Diketopiperazine of Homocysteine—20 mg. of sodium were dissolved in 25 cc. of dry liquid ammonia. To this solution 100 mg. of *l*-homocysteine diketopiperazine were added. This was immediately followed by the addition of 0.12 cc. of benzyl chloride. After the ammonia had been allowed to evaporate, the residue was suspended in 50 cc. of water and then filtered. The insoluble diketopiperazine of S-benzyl-*l*-homocysteine was recrystallized from 15 cc. of absolute alcohol. The short, thin prisms which melted at 183° weighed 60 mg. and gave a specific rotation of $[\alpha]_D^{25} = -61.0^\circ$ for a 1 per cent solution in pyridine. The following analysis was carried out.

$\text{C}_{12}\text{H}_{18}\text{N}_2\text{O}_2\text{S}_2$. Calculated, N 6.76; found, N 6.84

The *d*-sulfhydryl diketopiperazine prepared from *d*-homocysteine thiolactone hydrochloride was benzylated by the same method and the product obtained also melted at 183° and had an equal rotation of opposite sign to that of the *l* isomer. The following analytical value was found.

$C_{22}H_{26}N_2O_2S_2$. Calculated, N 6.76; found, N 6.91

Preparation of dl-Diketopiperazine of Homocysteine and of dl-Diketopiperazine of S-Benzylhomocysteine—10 mg. each of the *d*- and *l*-diketopiperazines of homocysteine were mixed and the combined amount recrystallized from absolute alcohol. The recrystallized product melted at 208° and crystallized in whetstones. There was no depression in the melting point of a mixture of this compound with the alcohol-soluble diketopiperazine (Compound B) prepared from *dl*-homocysteine thiolactone.

10 mg. of *d*- and 10 mg. of *l*-diketopiperazine of *S*-benzylhomocysteine were mixed and the mixture was recrystallized from alcohol. The compound melted at 165° and showed no depression in melting point when it was mixed with the benzyl derivative which had been prepared from the alcohol-soluble diketopiperazine of homocysteine (Compound B). The crystalline forms were identical.

From these results, we concluded that the diketopiperazine of *dl*-homocysteine which melts at 208° and which is soluble in alcohol is the racemic isomer. It gives a dibenzyl derivative which melts at 165°. The diketopiperazine which is insoluble in alcohol and which melts at 237° is the meso form. Its dibenzyl derivative melts at 176°.

Preparation of Polymer-Like Substance from l-Homocysteine Diketopiperazine with Hydrogen Peroxide—350 mg. of *l*-homocysteine diketopiperazine were dissolved in 60 cc. of hot water. H_2O_2 was added dropwise until the sulfhydryl test was negative. After standing for 10 minutes the amorphous precipitate which had separated was filtered. This amounted to 160 mg. Further evaporation of the filtrate gave 130 mg. more of amorphous product. The following analyses were obtained on the dry and ash-free basis (dried at 100° *in vacuo*).

Found, C 41.52, H 5.02, N 12.07, S 27.23

The polymer-like substance, which was prepared by oxidation with either air or H_2O_2 from the thiolactone or the sulfhydryl diketopiperazine, exhibited the same properties qualitatively.

Formation of Diketopiperazine of S-Benzylhomocysteine from Polymer-Like Material—500 mg. of the polymer-like amorphous material, which was prepared from *d*-homocysteine thiolactone hydrochloride by treating it with NaHCO_3 and by aerating the resulting solution, were added to 50 cc. of dry liquid ammonia which had 100 mg. of sodium in solution. 0.63 cc. of benzyl chloride was immediately added and the ammonia allowed to evaporate spontaneously. The residue was washed with water and then extracted with boiling absolute alcohol. The cooled extracts deposited 225 mg. of crystals which melted at 183° . The melting point showed no depression when the compound was mixed with the diketopiperazine of *S*-benzyl-*d*-homocysteine.

The amorphous product which was prepared from the *l*-homocysteine diketopiperazine by H_2O_2 oxidation was similarly reduced and benzylated in liquid ammonia. The recrystallized product likewise melted at 183° and showed no depression of the melting point when it was mixed with the diketopiperazine of *S*-benzyl-*l*-homocysteine.

Conversion of S-Benzyl-d-Homocysteine to Its Diketopiperazine—A suspension of 1 gm. of *d*-benzylhomocysteine in 25 cc. of CH_3OH was saturated with HCl . The solution was concentrated to an oil, CH_3OH was added, and the concentration was repeated. The material was then concentrated three times with CHCl_3 . The oily residue was dissolved in CHCl_3 and shaken with an aqueous suspension of Ag_2O freshly prepared from 3 gm. of AgNO_3 . The CHCl_3 layer was filtered through a small amount of norit and concentrated to an oil. This oil was heated at 70° for 16 hours. The mixture of dark oil and solid which resulted was treated with acetone and filtered. The residue, which weighed 25 mg., melted at 183° . Recrystallization from 5 cc. of boiling absolute alcohol failed to change the melting point of the compound. The specific rotation of the product was $[\alpha]_D^{26} = +62.0^\circ$ for a solution of 15.7 mg. in 5 cc. of pyridine. A mixture of the compound with the diketopiperazine of *S*-benzyl-*d*-homocysteine which was prepared from *d*-homocysteine thiolactone hydrochloride showed no de-

pression in melting point. The molecular weight was determined by the Rast method, with camphor as a solvent.

$C_{22}H_{32}N_2O_2S_2$. Calculated, mol. wt. 414; found, 410

SUMMARY

Homocysteine thiolactone hydroiodide when treated with dilute alkali followed by oxidation yields a high melting amorphous material which behaves like a polymer. An attempt has been made to establish the nature of this compound and to explain its formation. Hydrolysis of the polymer-like compound with strong acid yielded homocystine, thus indicating that the compound was some type of homocystine complex.

It was shown that when the thiolactone is freed from the hydroiodide it reacts with itself to give the diketopiperazine of homocysteine. The structure of the compound so formed was established by comparison of its S-benzyl derivative with the diketopiperazine of S-benzylhomocysteine which had been synthesized by accepted methods. When the thiolactone was opened quickly with an excess of strong alkali, formation of the diketopiperazine was prevented, and homocysteine was formed which could be isolated as homocystine after oxidation.

The opening of the thiolactone of benzoylhomocysteine with dilute alkali was also studied. Only benzoylhomocysteine was formed as would be expected.

The optically inactive mixture of the diketopiperazines of homocysteine was separated into the racemic and meso isomers. The identity of the racemic isomer was established by its preparation from the optically active diketopiperazines. This was confirmed by examination of the S-benzyl derivatives.

Evidence has been presented that the sulfhydryl diketopiperazine is an intermediate in the formation of the polymer-like substance, indicating the latter is either the dimeric or a polymeric disulfide of the diketopiperazine of homocysteine. Confirmatory evidence was obtained by reduction of the polymer-like substance in liquid ammonia with sodium followed by benzylation. The diketopiperazine of S-benzylhomocysteine was isolated from the reaction mixture.

The great insolubility of the polymer-like substance precluded

a molecular weight determination. This insolubility of the compound, its amorphous nature, and its dissimilarity in physical properties to the dimeric disulfide of the diketopiperazine of cystine favored the polymeric nature of this disulfide of the diketopiperazine of homocysteine.

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THE DISTRIBUTION OF UNCOMBINED HEXOSAMINE IN PINEAPPLE PLANTS SUPPLIED EITHER WITH AMMONIUM SULFATE OR CALCIUM NITRATE SALTS*

BY C. P. SIDERIS, H. Y. YOUNG, AND B. H. KRAUSS

*(From the Pineapple Experiment Station, University of Hawaii, Honolulu,
Hawaii)*

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Pineapple plants grown in solution cultures containing either ammonium or nitrate salts as sources of nitrogen were analyzed for various nitrogenous and carbohydrate constituents including hexosamine.

Hexosamine was identified by means of its reaction with *p*-dimethylaminobenzaldehyde after acetylation with acetylacetone according to the method of Elson and Morgan (4) as modified and adopted by various investigators (1-3, 6-10) and also by one of the authors (H. Y. Y.).

The method as finally adopted was as follows: Fresh plant tissues were thoroughly macerated in a porcelain mortar with quartz sand and the resulting pulpy material was extracted five times with water until a volume 10 times the weight of the tissues was obtained. The extract was treated immediately with lead acetate and then filtered. The filtrate was delead with dipotassium phosphate. It was then treated with toluene and used either immediately for chemical analysis or placed in a refrigerator for future use. The reagents were prepared as described by Palmer, Smyth, and Meyer (10). 1 cc. of plant extract was delivered into a 16 × 150 mm. Pyrex test-tube calibrated at 5 cc. 0.5 cc. of acetylacetone solution was introduced and the tube was either covered with a glass bulb or lightly stoppered with a cork stopper. The mixture was digested in a boiling

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water bath for 15 minutes. It was then cooled to room temperature and diluted with 2.5 cc. of alcohol. 0.5 cc. of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) was added drop by drop with shaking and the mixture was brought to the 5 cc. mark with alcohol. After precisely 20 minutes standing at room temperature, the extinction coefficient was read in a Pulfrich gradation photometer (Zeiss) with use of a 1 cm. cell and Filter S-53. The amount of hexosamine (glucosamine) present was calculated by the equation,

$$\frac{\text{Extinction coefficient}}{0.0066} = \text{micrograms hexosamine}$$

The only important modification of the method of Palmer *et al.* (10) is in the time allowed for the development of the final color. A reaction time of 20 minutes at room temperature was chosen because it was found that in the presence of large amounts of reducing sugars, such as are found in pineapple extracts, serious error arose from interference by the sugars when longer periods of reaction were allowed. Data are presented in Table I to bring out this point. 2 mg. of sugar per cc. were employed, which was approximately the maximum amount of reducing sugar present per cc. of extract prepared by our procedure. As noted in Table I, even after 20 minutes reaction, a slight color developed with most of the sugars. Therefore, a small correction, based on the values given in Table I, was made, depending on the time of reaction and the kind and quantity of sugar present. The non-interference of different sugars as reported by others (4, 10) is probably due to the presence of very small amounts in their samples.

Satisfactory recovery of hexosamine is obtained when glucosamine hydrochloride is added either to plant extracts or to invert sugar, according to Table II.

Asparagine, sodium acetate, and dipotassium hydrogen phosphate do not produce any color, whereas pure hexosamine develops a fully complete color even after 10 minutes. The amino acids, alanine, arginine, cystine, glutamic acid, glycine, histidine, leucine, lysine, methionine, proline, serine, tryptophane, tyrosine, and valine, when present alone, did not produce any color. Only

glycine and lysine, when present with a large quantity of a reducing sugar such as glucose, produced a measurable amount of color. This was also true of a mixture of ammonium sulfate and glucose, as shown in Table III. However, the addition of glucose and

TABLE I

Effect of Time on Color Developed by Various Substances in Determination of Hexosamine

	mg. per cc.	Extinction coefficients					
		10 min.	20 min.	30 min.	45 min.	1 hr.	1.5 hrs.
Glucosamine HCl.....	0.050	0.270	0.270	0.270	0.268		0.270
Dextrose.....	2.00	0.001	0.008	0.020	0.035	0.060	0.100
Levulose.....	2.00	0.010	0.020	0.042	0.075	0.110	0.150
Mannose.....	2.00		0.016				
Sucrose.....	2.00	0	0	0.005	0.003	0.005	
Invert sugar.....	2.00	0	0.015	0.033	0.047	0.075	
Plant Extract C1.....	1	0.080	0.085	0.102			0.173
“ “ C5.....	1	0.273	0.287	0.290			0.320

TABLE II

Recovery of Glucosamine Hydrochloride Added to Aqueous Plant Extracts and Solution of Reducing Sugar

Sample	Glucosamine				Recovery
	Present	Added	Total found	Recovered	
	micrograms	micrograms	micrograms	micrograms	per cent
Plant Extract C1*.....	15	50	63	48	96
“ “ C1.....	15	50	63	48	96
“ “ C5.....	52	25	77	25	100
“ “ C5.....	52	25	80	28	112
Invert sugar.....	0	50	47	47	94

ammonium sulfate to pure glucosamine did not increase the color developed by glucosamine alone, as found by Palmer *et al.* (10). Furthermore, the addition of glycine, lysine, glucose, and ammonium sulfate to glucosamine did not influence the color produced by glucosamine except in cases where hexosamine was

present in very low concentrations. In view of the fact, based on quantities of α -amino nitrogen, that our samples could not possibly contain any higher concentrations of either glycine or lysine than those added to them and that glucosamine, when added to these samples, was satisfactorily recovered, it is unlikely that interfering substances were present to any greater extent than as indicated in Table III.

The calibration curve which was prepared with pure glucosamine hydrochloride shows practically a straight line up to 0.080 mg.

TABLE III

Effects of Glucose, Ammonium Sulfate, Glycine, and Lysine Added to Solutions of Hexosamine on Extinction Coefficient Values of the Latter

Sample No.	Hexosamine	Glucose	(NH ₄) ₂ SO ₄	Glycine	Lysine	Extinction coefficient
	micrograms per cc.	mg. per cc.	micrograms per cc.	micrograms per cc.	micrograms per cc.	
1	10	0	0	0	0	0.058
2	25	0	0	0	0	0.139
3	50	0	0	0	0	0.282
A	0	2	10	0	0	0.032
B	0	2	0	20	0	0.040
C	0	2	0	0	20	0.052
D	0	2	0	20	20	0.058
1-E	10	2	10	20	20	0.103
2-F	25	2	10	20	20	0.170
3-G	25	2	10	500	0	0.238
4-H	50	2	10	0	0	0.275
5-I	50	2	10	20	0	0.280
6-J	50	2	10	0	20	0.275
7-K	50	2	10	20	20	0.264

per cc. Higher concentrations of hexosamine than 0.08 mg. per cc. are subject to an appreciable error in its determination by this method. It was, therefore, possible to calculate from values obtained from known concentrations, some of which are shown in Table III, the extinction coefficient factor 0.0055 per microgram of glucosamine hydrochloride or 0.0066 per microgram of glucosamine which was employed in the equation given in the above procedure.

The distribution of hexosamine in plants has been studied very little. Scholl (11) and Iwanow (5) have reported its presence in a

nitrogen-containing polysaccharide (chitin) occurring in the cell walls of higher fungi. There is either very little or possibly no information regarding the distribution of hexosamine in chlorophyllous plants. And as far as the authors are acquainted with the

TABLE IV

Mg. of Hexosamine per Gm. of Fresh Tissues of Different Sections of Leaves, Stem, and Roots of Pineapple Plants Grown in Solution Cultures Supplied Either with Ammonium Sulfate or Calcium Nitrate Salts As Nitrogen Source

Plant sections		Nitrogen supply	
		Ammonium sulfate	Calcium nitrate
Old leaves	Basal and transitional	0.191	0.056
	Chlorophyllous, low	0.278	0.082
	“ intermediate	0.445	0.118
	“ terminal	0.455	0.137
Mature leaves	Basal	0.155	0.100
	Transitional	0.228	0.164
	Chlorophyllous, low	0.260	0.254
	“ intermediate	0.330	0.236
	“ terminal	0.391	0.236
Active “	Basal	0.158	0.109
	Transitional	0.155	0.118
	Chlorophyllous, low		0.246
	“ intermediate	0.256	0.264
	“ terminal	0.346	0.336
Young “	Basal	0.127	0.104
	Transitional	0.071	0.145
	Chlorophyllous, low	0.164	0.224
	“ intermediate and terminal	0.288	0.282
Roots		0.009	0.009
Stem	Base	0.142	0.082
	Middle	0.045	0.045
	Apex	0.049	0.082

literature, free occurring hexosamine, *e.g.* not obtained from hydrolyzed glycoproteins or polysaccharides, has not been reported in plants. Only in the cysts or echinococci of *Tænia echinococcus* (12) was hexosamine found in the uncombined state.

The distribution of free hexosamine in different sections of

pineapple leaves, roots, and stem of two sets of 1 year-old pineapple plants, one grown in ammonium sulfate and the other in calcium nitrate solution cultures, is reported in Table IV. The data show that the mature and advanced groups of leaves of plants supplied with ammonium sulfate contained more hexosamine than comparable sections of the leaves of plants supplied with calcium nitrate. The active and young groups of leaves of both lots of plants contained, with certain exceptions, approximately equal amounts of hexosamine. The physiological significance of these findings together with other data will be presented and discussed in a future publication.

Studies are under way for the isolation and further identification of the above substance by means of other chemical reactions.

SUMMARY

The method of Elson and Morgan (4) for the determination of hexosamine with the modifications of various investigators and the authors has been employed for the determination of free or uncombined quantities (not obtained from hydrolyzed glycoproteins) of this substance in pineapple plant tissues and found satisfactory. Possible interference by glycine and lysine in hexosamine determinations is indicated.

The modification as introduced by one of the authors (H. Y. Y.) limits the time to 20 minutes between heating and reading the color value in a Pulfrich photometer, because it was found that with longer time intervals reducing sugars (2 mg. per cc.) interfered with the accuracy of the method.

Hexosamine was present in considerably greater amounts in the advanced and mature leaves of plants supplied with ammonium sulfate than in those of plants supplied with calcium nitrate. In the active and young leaves of both lots of plants the amounts of hexosamine were approximately the same.

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A NEW VITAMIN D IN COD LIVER OIL

By CHARLES E. BILLS, O. N. MASSENGALE, K. C. D. HICKMAN,
AND E. LEB. GRAY

(From the Research Laboratory, Mead Johnson and Company, Evansville,
Indiana, and the Research Laboratory, Eastman Kodak Company,
Rochester, New York)

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In previous studies (1) it was shown that the liver oils of different species of fish are unequally effective, per rat unit, for the prevention of rickets in chickens. The experiments indicated the existence of more than one kind of vitamin D in the oils. More recently it was found (2, 3) that commercial cod liver oil, when subjected to molecular distillation, gives physical evidence of containing two principal, and two minor vitamins D, with traces of still two more forms.

The present report covers the bioassay of the most volatile vitamin D of cod liver oil. This is one of the minor forms. It was separated (2, 3) in the Eastman laboratory as the first fraction of the first fraction of the vitamin D-bearing distillate obtained from 2 tons of commercial Norwegian cod liver oil. The bioassay techniques for rats and for chickens, with procedures for estimating errors, are also elsewhere described (4, 5). The rat assay technique is in a somewhat improved form, and includes the use of a reference oil such as is used in the chicken technique.

The cod liver oil was assayed against the U.S.P. reference cod liver oil. 50 rats were used in the final assembly, twenty-five on the test oil and twenty-five on the reference, other rats used in exploratory trial assays not being counted. The oil contained 270 international units of vitamin D per gm. Since the liver oil of the common codfish, *Gadus morrhua*, contains on the average 100 international units per gm., and rarely more than 150 international units, it is reasonable to suppose that this inordinately potent oil represented some codfishes other than *Gadus morrhua*.

It may be noted that oils from the allied species of *Gadus* are legally cod liver oil, and in fact are typical of the better medicinal grades of this product.

The distillate was assayed similarly, with twenty-three pairs

TABLE I

Relative Effectiveness of Cod Liver Oil and Low Boiling Distilled Vitamin D for Rats and Chickens

Product administered to chickens	Amount per 100 gm. diet		Response obtained			Efficacy ratio	
		Probable error	Femur ash	I. U. of D per 100 gm.	Probable error	Cod liver oil = 100	Probable error
	inter-national units	per cent	per cent		per cent		per cent
Theoretical responses, taken from master curve, pure cod liver oil	6.67 8.00 16.00 60.00		40.27 41.50 45.77 >48.30			100 100 100 100	
Series 1							
U.S.P. reference cod liver oil	8.00 ± 2.0 8.00 ± 2.0		40.50 40.24	6.90 6.64	± 9 ± 10	86 83	± 9 ± 10
Commercial cod liver oil	8.00 ± 6.0 8.00 ± 6.0		39.82 38.90	6.12 5.20	± 10 ± 11	77 65	± 12 ± 13
Low boiling D distillate	6.67 ± 7.0 6.67 ± 7.0		35.50 35.70	0.01 1.70	Great "	* *	* *
Series 2							
U.S.P. reference cod liver oil	8.00 ± 2.0 8.00 ± 2.0		41.13 41.55	7.63 8.05	± 9 ± 9	95 101	± 9 ± 9
Low boiling D distillate	16.00 ± 7.0 16.00 ± 7.0 60.00 ± 7.0 60.00 ± 7.0		42.93 41.14 45.29 46.22	9.75 7.64 14.68 17.48	+10, -9 ± 9 ± 9 +10, -9	61 48 24 29	+12, -11 ± 11 ± 11 +12, -11

¹ Response too low for significant interpretation.

of rats. It contained 2666 international units of vitamin D per gm. Thus it represented a potency concentration of about 10 times, though it was still remote from a pure vitamin D.

The assays with chickens were carried out in two series, which are summarized in Table I. For each test group in each series,

ten chickens were used, and each test group was assayed twice, for better accuracy. Also given in Table I, are the theoretical values, taken from the master curves in the published technique (5), which illustrate the ideal, or expected, femur ash development in response to doses of pure cod liver oil of the same size as the doses of the preparations investigated.

In Series 1 the reference oil gave a response which was somewhat below par, but not significantly so. The commercial oil gave a still lower response, but here again the difference was not significant, if one accepts as the criterion of significance the limit of 3 times the probable error. The low boiling vitamin D, however, failed to elicit femur ash response, although it was administered at a level at which good response was expected.

In Series 2, the reference oil gave a response close to normal. 16 units of low boiling vitamin D had an efficacy ratio of about half normal, and 60 units about one-quarter normal. These are significant differences. The fact that the efficacy ratios at 16 units and 60 units were not the same merely indicates, as has been pointed out before in another connection (5), that the response to the vitamins involved is dissimilar. It is additional evidence that the low boiling distilled vitamin is different from the bulk of the vitamin in the original oil.

In the paper (3) in which the distillation of the several vitamins D from cod liver oil was described, the point was made that the lowest boiling form may differ from the familiar forms in the complete absence of the side chain on the cholane nucleus. This supposition is based only on boiling point analogies under the conditions of molecular distillation, but if it is true, the form of vitamin D investigated in the present work would be the simplest possible one, and in a sense, the prototype of all the others.

SUMMARY

The most volatile of several vitamins D which occur in cod liver oil was assayed with rats and chickens. Per rat unit, it was from one-half to one-fourth as effective for chickens as the total vitamin D of the oil. The assays substantiate the evidence, previously obtained from molecular distillations, that cod liver oil contains several vitamins D.

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PROTEOLYTIC ACTIVITY OF PLASMA DURING TUMOR OR EMBRYONIC GROWTH IN RATS

BY LEOPOLD WEIL AND MARY A. RUSSELL

(From the Biochemical Research Foundation of the Franklin Institute, Philadelphia)

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Tumor and embryonic growth are characterized by rapid cell division, which exerts a marked influence on certain metabolic processes. Both involve, among other things, an intensive protein metabolism for building up the protein constituents of the cell. It seemed probable that such growth processes would be reflected in some way in the proteolytic system of the blood of the animal involved. Such a relationship is here demonstrated.

EXPERIMENTAL

Because of individual variations, it is important, in studies of this kind, that blood changes be followed in individual animals throughout the whole growth period. Rats are particularly suitable subjects, since they can be held under closely standardized conditions, and since they are readily susceptible to transplantable tumors which grow at a rate sufficiently rapid so that results may be obtained in a relatively short time. However, rats are subject to the disadvantage of a limited blood supply for repeated enzymatic determinations. The total amount of blood in a 200 gm. rat is only 6 to 7 cc., so that when determinations are to be made several times weekly over the whole period of tumor or embryonic growth, the quantity taken for each analysis should not exceed 1 or 2 drops. Removal of larger amounts involves the risk of upsetting the balance of the blood enzyme systems. This difficulty was overcome by making use of the excellent microtechnique of Linderstrøm-Lang and Holter (1) which, with certain modifications, made it possible to carry out the desired analyses with only 0.04 cc. of blood.

The general technique was as follows: The rats were fasted for about 18 hours before an analysis was to be made. The tail was then dipped into water at about 50° to cause expansion of the tail vein, thus enhancing subsequent bleeding. A small section of the tail was cut off, and 2 drops of blood were squeezed out onto a microscope slide. The blood was drawn up into a paraffin-washed capillary tube (calibrated roughly to 40 c.mm.) and quickly blown out onto a microscope depression slide containing the necessary amount of potassium oxalate in dry form to prevent coagulation. The oxalated slides were prepared by pipetting 30 c.mm. of 0.45 per cent potassium oxalate solution into the depression, and drying at 110°. The oxalated blood was drawn up into thick walled capillary tubes, about 7 cm. long and 1 mm. inside diameter, similar to those used in the determination of red blood cell sedimentation. The tubes were tightly closed by means of rubber bands, and placed in a small centrifuge at 3000 R.P.M. for 5 minutes. The clear, unhemolyzed plasma was then carefully removed by a capillary pipette, which also was paraffined to reduce loss of plasma due to cohesion at the capillary wall. (The paraffin can best be applied by rinsing the capillary tubes once with a solution of paraffin in toluene, and drying at 110°. The thin coating left on the walls gives the desired effect.) The plasma was again blown out on a slide, and was then ready for exact measurement for the enzyme determinations. For this, the technique of Linderstrøm-Lang and Holter was followed in detail. To prevent errors due to evaporation, all manipulations were carried out as rapidly as possible. In all studies to be reported, 7 c.mm. of plasma were used for each enzyme determination.

pH Optimum of Plasma Aminopolypeptidase—Preliminary studies were made on the pH optimum of plasma aminopolypeptidase. In agreement with Grassmann and Heyde (2) an optimum at pH 7.5 was found. Reaction mixtures were made to contain 1 cc. of pooled oxalated rat plasma, 3 cc. of citrate-phosphate buffer (3), and 0.5 cc. of 0.2 M *dl*-leucylglycylglycine previously adjusted to the pH of the buffer. After addition of a drop of toluene, the mixtures were incubated at 37° for 20 hours. 8 cc. of water were added, and the protein precipitated by slowly dropping in 1 cc. of 5 per cent colloidal Fe(OH)₃ at about 90°.

After the material was centrifuged and filtered, the increase in free amino groups was measured on 7 cc. of the filtrate by the Van Slyke method. The latter permits the use of buffers, which interfere in the microtitration method. The results, expressed as cc. of 0.05 N KOH equivalent to the amino nitrogen found, are as follows:

6.0 pH	6.5 pH	7.0 pH	7.5 pH	8.0 pH	8.5 pH
Activity, cc. 0.05 N KOH					
0.32	0.36	0.40	0.68	0.56	0.32

Attempts were made to measure the dipeptidase of the plasma, but the activities found were so low that they were hardly outside the limits of the experimental error. The instability of this enzyme, as reported by Grassmann and Heyde (2), may account for this observation. It is probable, therefore, that the decomposition of *dl*-leucylglycylglycine is almost wholly due to the action of the plasma aminopolypeptidase, and that the above data truly represent the activity of this enzyme.

Aminopolypeptidase in Rat Plasma during Tumor Growth—Normal rats and rats inoculated with Walker No. 256 carcinoma and Philadelphia No. 1 sarcoma were studied. Blood samples were taken every few days, as described above, beginning before the inoculations were made, and continuing until the last stages of tumor growth. Tumor tissue was implanted subcutaneously into each flank, the twin tumors, in general, growing at about the same rate. In case the inoculations were unsuccessful, they were repeated; if tumor growth again failed to occur, the rats were considered to be immune (negative). For the aminopolypeptidase estimation, 7 c.mm. each of rat plasma, 0.2 M *dl*-leucylglycylglycine (pH 7.5), and 0.005 N H₂SO₄ were pipetted into a microreaction vessel. This amount of acid kept the reaction mixtures very near to pH 7.5 over a period of 48 hours, and made unnecessary the use of buffers, which have a disturbing effect on the subsequent titration. The reaction vessels were placed in a desiccator containing a small vial of chloroform to stop bacterial growth. A constant chloroform vapor pressure (10 mm. of Hg) was maintained by means of a mercury regulator. To prevent

evaporation of the reaction mixtures, water was placed at the bottom of the desiccator. Incubation was carried out for 22 hours at 37°. After addition of 50 c.mm. of 0.005 N MgSO_4 (to enhance flocculation of the colloidal $\text{Fe}(\text{OH})_3$), the proteins were precipitated by means of 7 c.mm. of 5 per cent colloidal $\text{Fe}(\text{OH})_3$. The latter must be added very slowly, which can be done by decreasing the pressure used for emptying the micro-pipette (see Linderstrøm-Lang and Holter (1)); proper stirring during the addition was obtained by means of the usual magnetic

TABLE I

Aminopolypeptidase Activity of Rat Plasma during Tumor Growth

The values are given in terms of c.mm. of 0.05 N alkali.

Days after inoculation	Normal rats				Philadelphia No. 1 sarcoma				Walker No. 256 carcinoma				Tumor-resistant rats	
0	4.58	6.14	4.98	4.10	4.54	4.64	4.50	4.92	5.12	5.36	4.98	5.24	5.08	4.00
2	4.38	4.62	6.12	4.84	5.02	4.42	4.54	4.98	5.22	6.14	5.54	5.72	5.48	4.96
5	4.56	4.64	4.60	4.58	4.62	4.36	5.96	5.66		6.14	5.78	5.82	4.74	
8	4.70	4.62	4.90	4.38	4.88	4.24	4.56	4.16	5.68	4.66	5.58	5.36	4.58	4.40
12	5.64	5.64	5.34	5.78	6.16	6.30	5.86	4.90	4.66	4.56	5.66	5.16	5.02	5.40
15	6.42	5.23	5.44	6.16	5.44	6.10	4.46	5.54	4.96	5.02	4.80	4.72	6.16	5.34
19	5.84	5.34	5.38	5.68	5.60	5.28	6.24	5.56	4.58	4.92	4.92	4.64		5.58
22	5.54	4.60	5.78	5.76	5.92	5.68	5.56		4.86	5.12	5.20	4.96	5.96	5.66
24	5.20	4.98	5.62	5.40	5.62	5.20	4.96	4.70	5.84	5.44	5.56	6.06	5.34	5.32
28	5.12	4.38	5.76	4.88	4.94	3.94	4.76	4.54	5.10	5.63	4.82	5.36	5.14	4.90
32	5.64	5.02	5.08	5.20	4.96	5.40	5.12	5.86					5.48	5.00
36	5.06	5.28	6.54	5.42	5.34	5.88	6.02	5.58					5.50	5.16
41	4.98	5.66	5.14	5.98	4.96	5.54	5.28	4.90					5.60	5.52
45	5.60	4.96	5.44	5.90	6.02	5.60	5.70	5.10					5.04	5.00

stirring device. The reaction vessels were then closed with a rubber cap and allowed to stand 2 hours in the ice box. At this time the supernatant fluid had become clear and could be centrifuged. The $-\text{COOH}$ increase in 35 c. mm. of the supernatant liquid was measured by microformol titration (4). To prevent the disturbing effect of atmospheric CO_2 during the alkali titration, the previous method of Linderstrøm-Lang, Weil, and Holter (5) was modified by placing a layer of paraffin oil above the reaction mixture. This technique is simpler and more effective than that previously used. Table I gives the aminopolypeptidase

activities found in plasma, in terms of c.mm. of 0.05 N alkali equivalent to the —COOH groups liberated. It is evident that no important changes in this enzyme took place during tumor growth. Rats bearing Philadelphia No. 1 sarcoma or Walker No. 256 carcinoma showed activities quite similar to those of normal or tumor-resistant rats.

Plasma Proteinase

The literature on plasma and serum proteinase is quite extensive. Hedin (6), Okubo (7), Utkin-Ljubowzow (8), Abderhalden (9), and Schmitz (10) were able to demonstrate a proteinase in serum active near neutrality. In addition, Utkin-Ljubowzow (8) found a pepsinase having an optimum at pH

TABLE II
pH Optimum of Plasma and Serum Proteinase in Cc. of 0.05 N KOH

pH.....	3	4	5	6	7	8
Plasma, measured on gelatin.....	0.0	0.0	0.10	0.32	0.82	0.24
Serum, " " ".....	0.0	0.0	0.15	0.54	1.28	0.40
Plasma, " " clupein sulfate.....	0.0	0.0	0.0	0.22	0.46	0.26

2.6. Kleinemann and Scharr (11), however, claim that blood serum, like cathepsin, exhibits its proteolytic activity in acid medium, and that only the globulin fraction shows activity at pH 7 (casein used as substrate). Our results indicate that plasma and serum contain a proteinase with an optimum near pH 7; no activity could be detected at the more acid pH values.

pH Optimum of Serum and Plasma Proteinase—Serum and plasma were prepared from a sample of rabbit blood. To 1 cc. portions were added 1 cc. of water, 3 cc. of citrate-phosphate buffer, and 1 cc. of 6 per cent gelatin or 1 cc. of 6 per cent clupein sulfate, these substrates being previously adjusted to the proper pH. After incubation at 37° for 42 hours, 4 cc. of water were added, and the mixtures deproteinized with 1 cc. of 5 per cent colloidal Fe(OH)_3 . The —NH_2 increases in 6 cc. of the filtrates thus obtained were determined by the Van Slyke method. The results are given in Table II. As shown, plasma and serum

proteinases exhibit their optimal activities at neutral pH. It will be noted that serum proteinase is slightly more active than is plasma proteinase. The recent work of Schmitz (10) demonstrated the presence, in blood, of a proteinase inhibitor, and it is possible that coagulation may cause a partial removal of this inhibitor, thus accounting for the greater activity of the serum proteinase.

Effect of Cysteine on Plasma Proteinase Activity—It appeared possible that plasma might contain a catheptic enzyme, but in an inactive form. This possibility was investigated by use of cysteine as activator for this enzyme. The experiments were carried out as described for the determination of the pH optimum, except that 5 mg. of cysteine hydrochloride, adjusted to the proper pH, were used in addition. Gelatin was used as substrate. To prevent oxidation of cysteine, the incubations were carried out in an atmosphere of hydrogen. Chloroform was used as antiseptic. As shown in the following tabulation, no catheptic activity could be detected with cysteine as activator, the proteinase itself being almost wholly inhibited at pH 7.

pH.....	3	4	5	6	7	8
Initial activity.....	0.0	0.0	0.0	0.30	1.02	0.10
Activity with cysteine.....	0.0	0.0	0.0	0.0	0.08	0.0

Plasma Proteinase Activity during Tumor Growth in Rats—Normal rats, rats with Philadelphia No. 1 sarcoma, Jensen sarcoma, and Walker No. 256 carcinoma, and rats resistant to tumor growth after repeated inoculation were studied. The technique was identical with that used in the aminopolypeptidase work, except that 7 c.mm. of 0.006 N H₂SO₄ were used to maintain the pH at 7, and that 7 c.mm. of 5 per cent casein were used as substrate instead of the *DL*-leucylglycylglycine. Incubation was carried out at 37° for 44 hours, with the precautions previously described. The results are given in Table III, in c.mm. of 0.05 N alkali equivalent to the —COOH increase. As shown in all cases studied, tumor growth is associated with a decrease in plasma proteinase activity. The decrease is noticeable even at an early stage, and after reaching a minimum value in 15 to 18 days,

the activity remains fairly constant. Normal and tumor-resistant rats behaved similarly in showing a practically constant level of serum proteinase activity throughout the whole experimental period.

Effect of Tumor Removal on Plasma Proteinase Activity—Since tumor growth appeared to be related to a decrease in plasma proteinase activity, it was of interest to determine whether opera-

TABLE III
Plasma Proteinase Activity during Tumor Growth in Rats
The values are given in terms of c.mm. of 0.05 N alkali.

Days after inoculation	Normal rats				Philadelphia No. 1 sarcoma ^a				Walker No. 256 carcinoma		Jensen sarcoma		Tumor-resistant rats					
0	1.98		1.98	1.92	2.10	2.02	1.98	1.98	2.32	2.20	2.20		1.86	1.80	1.98	1.90		
3	2.12	2.06	1.82	1.90	1.66	2.08	1.64	1.96	1.80				1.94	1.84	2.28	1.92		
8	1.80	1.98	2.08	2.04		1.90	1.52	1.64	1.52	1.42			1.84	1.72	2.26	1.96		
11	1.98	1.90	1.98	1.66	1.74	1.34	1.62	1.64	1.42	1.50	1.80	2.04	1.96	1.72				
15	1.96	1.96	1.88	1.94	1.70	1.56	1.66	1.52	1.42	1.48	1.98				1.76	1.84		
18	1.98	2.08	1.96	1.98	1.68	1.54	1.42	1.50	1.34	1.50		1.94	2.20			2.08		
22	1.94	1.96	1.94	1.92	1.52	1.40	1.30	1.54	1.22	1.32	2.12	1.82	1.84	1.98	1.90			
28	2.26	1.98	2.38	1.98	1.40	1.42	1.37	1.40	1.52	1.50	2.24	1.92	1.94	2.22	2.30			
31	1.84		2.38	2.30	1.58	1.52	1.56	1.64	1.50	1.60	2.32	2.08	1.86	2.30	2.38			
36					1.66	1.60	1.38	1.56	1.50	1.62	2.00	2.22	2.26	2.40	1.98			
39	1.98	1.80	2.02	2.10	1.52	1.44	1.48	1.40	1.60	1.54	1.90		2.02	2.32	2.10			
43	2.28	2.00	2.10	1.96	*	*	1.16	*	1.40	1.36	2.06			1.82				
47	2.30	2.02	2.00	2.10			*		*	1.30		1.96		1.92	1.84			
51			2.10							*	1.98							
54	2.02		2.12								1.96		2.00	2.03				
58	1.98	2.20	2.08	1.97							2.16	2.02	1.90		2.12			

* The animal died.

tive removal of the tumor would result in restoration of the normal level. Rats were inoculated with Philadelphia No. 1 sarcoma, and estimations of the proteinase activity made during the first 40 days of growth. The tumors were then removed by operation, the enzyme estimations being continued. As shown in Fig. 1, the proteinase activity decreased steadily, as before, but was quickly restored to normal levels after tumor removal. Whether the decrease in proteinase activity is due to an increase

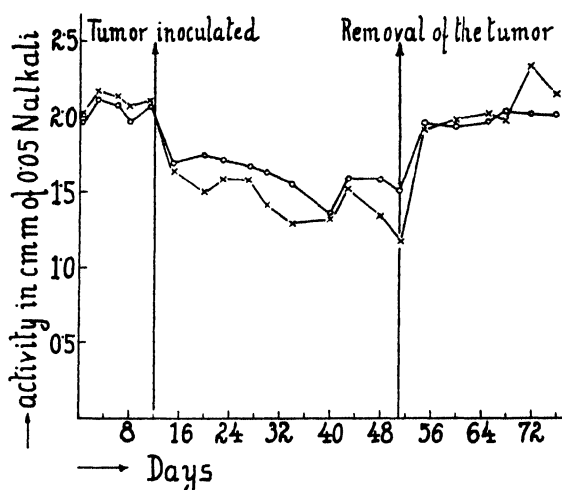


FIG. 1. The effect of successful tumor removal upon the plasma proteinase activity in the rat.

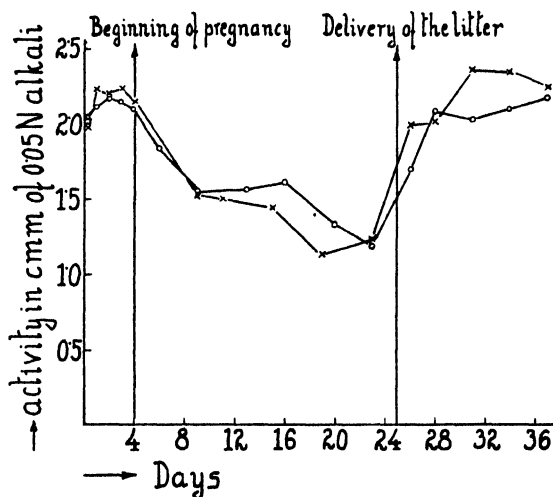


FIG. 2. Relation of plasma proteinase activity to pregnancy

of inhibitor formation (10), or represents an actual decrease in enzyme concentration, as reported for humans by Herzfeld and Klinger (12) cannot at present be decided.

Relation of Plasma Proteinase Activity to Pregnancy—The question whether pregnancy would give a similar picture, with respect to plasma proteinase activity, as was found for tumor growth, was next studied. Blood samples from female rats were measured for proteinase activity, as previously described, beginning before mating and continuing after delivery of the litter. The start of pregnancy was calculated by subtracting 21 days from the delivery date. Normal female rats show values for plasma proteinase activity similar to those of normal males. As shown in Fig. 2, 4 or 5 days after pregnancy begins the value for proteinase activity drops and reaches a minimum shortly before delivery. Almost immediately afterwards the activity begins to increase again, and reaches a normal value in a few days. The similarity in the results obtained during tumor growth and in pregnancy indicates that the observed phenomena are in some way connected with rapid tissue growth. This relationship is emphasized by the rapid return to normal values of proteinase activity after elimination of the cause (tumor removal or termination of pregnancy).

SUMMARY

1. Micromethods for the estimation of peptidase and proteinase activities in a few drops of blood are described.

2. The aminopolypeptidase activity of rat plasma remains constant during the growth of transplantable rat tumors. The enzyme has an optimum at pH 7.5.

3. Plasma and serum proteinases exhibit optimal activity at pH 7. Serum has a slightly higher activity than plasma prepared from the same blood.

4. During tumor growth or pregnancy in rats, the plasma proteinase activity drops considerably. When the tumor is removed by operation, or after termination of pregnancy, the proteinase activity rapidly returns to the normal level. The similarity of these effects in tumor growth and pregnancy is emphasized.

5. Normal and tumor-resistant rats show constant plasma proteinase and aminopolypeptidase activities over long periods of time.

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THE FAT ACIDS IN THE LECITHIN AND GLYCERIDE FRACTIONS OF EGG YOLK

By R. W. RIEMENSCHNEIDER, N. R. ELLIS, AND HARRY W. TITUS

(From the Animal Nutrition Division, Bureau of Animal Industry, United States Department of Agriculture, Washington)

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Approximately two-thirds of the dry matter of egg yolk consists of materials extractable with absolute alcohol. According to MacLean and MacLean (1), observations of nearly 100 years ago showed the presence of phosphorus-containing fats in egg yolk. There followed several studies which gradually established the presence of a number of lipids each containing one or more fat acids. Chief interest has centered around egg yolk lecithins. Besides identifying stearic, palmitic, oleic, and linoleic acids, as reported by earlier workers (1), Levene and Rolf (2) found traces of an acid containing 20 carbon atoms and four double bonds which they identified as arachidonic acid. Hatakeyama (3), in 1930, reported margaric, arachidonic, and oleic acids, while Yokoyama (4), in 1935, reported isopalmitic, oleic, and clupanodonic acids. It is obvious, therefore, that even the more recent work has not entirely decided the fat acid composition of egg yolk lecithin.

References to the fat acid composition of the neutral egg yolk glycerides, freed of N- and P-containing substances, are extremely limited. However, Suzuki (5) found the egg yolk oil (presumably a mixture of glycerides and phospholipids) from Chinese hen's eggs contained isopalmitic, palmitic, oleic, and small amounts of linoleic acids, whereas the oil from white Leghorn hen's eggs contained, in addition, about 4 per cent of stearic acid and small amounts of arachidonic acid.

The present investigation was undertaken for the purpose of obtaining more complete information than was available on the fat acid components in the egg yolk lipids obtained from hens

kept on a representative diet of known composition and high nutritive value. The lipids of the glyceride and lecithin fractions have been studied. The fractions, presumably of cephalin and spingomyelin, which were obtained in the course of purifying the two main fractions were not further analyzed for fat acid components because of the small yields. The fat acids of the glyceride and lecithin fractions were separated by fractional distillation of their methyl esters; the analysis of the distilled fractions formed the basis for the estimation of the component acids, subject in each case to confirmatory evidence of identity.

EXPERIMENTAL

Source of Sample—Approximately 300 eggs were obtained in February, 1936, from a group of yearling hens from a cross of Rhode Island Red males with barred Plymouth Rock females which had been fed for several months on a ration made up as follows: basal feed mixture 75.43 per cent (composed of ground yellow corn 52.63 per cent, red dog flour 25.79 per cent, rolled oats 15.79 per cent, and alfalfa leaf meal 5.79 per cent), meat and bone scrap 8 per cent, North Atlantic fish-meal 7 per cent, dried skim milk 5 per cent, mineral supplements 4.57 per cent, and cod liver oil 2 per cent.

Preparation of Lecithin and Glycerides—4.5 kilos of fresh egg yolks were extracted with cold acetone and ether, and with warm alcohol. The lecithin was separated and purified by a combination of the acetone precipitation and cadmium salt methods used by Levene and Rolf (6), Yokoyama (4), and others. The purified product (323 gm.) was found to contain 1.68 per cent nitrogen and 3.91 per cent phosphorus.

The remaining lipid material after removal of the bulk of the phospholipids was dissolved in absolute alcohol and the glycerides precipitated by storage at -15° to -10° . Most of the supernatant alcohol solution was siphoned off, after which the remainder was filtered under suction. After this process had been repeated five times, the precipitated glycerides gave negative tests for phosphorus and nitrogen. The final yield of purified glycerides was 558 gm.

Distillation and Analysis of Methyl Esters of Fat Acids from Lecithin—191 gm. of fat acids were recovered from the saponifica-

tion of 300 gm. of lecithin. The saponification equivalent of these mixed acids was found to be 278.6; the iodine number 103.2. The methyl esters were prepared in the usual manner with methyl alcohol and dry HCl gas. The esters were then distilled through an electrically heated column (7) and a series of ten fractions collected. The analyses of the fractions are given in Table I.

TABLE I

Yields and Characteristics of Fractions Obtained from Distillation of Methyl Esters of Fat Acids from Lecithin

Fraction No.	Weight	B.p., 1.0-0.5 mm.	Mean mol. wt.	I No. (Wijs)
	gm.	°C.		
1	3.60	125-130	268.1	11.31
2	32.50	130	270.8	13.27
3	15.10	130-132	280.1	35.78
4	13.50	132-133	284.2	48.74
5	17.95	133-135	288.2	61.90
6	33.30	135-137	291.1	75.50
7	25.60	137-140	296.7	90.40
8	20.00	140-145	299.8	127.30
9	18.05	145-155	329.4	357.00
10	4.85*	Residue		

* The residue was deeply colored, indicating some effect of heating.

TABLE II

Lead Salt-Ether Separations of Composites A and B of Lecithin Fat Acids

Composite	Solid acid fraction		Liquid acid fraction	
	Mean mol. wt.	Iodine No.	Mean mol. wt.	Iodine No.
A	258.5	3.67	279.3	104.2
B	261.1	5.05	283.8	104.6

Fractions 1 and 2 were combined to form Composite A and Fractions 3, 4, 5, and 6 were combined to form Composite B. The two composites were subjected to lead salt-ether separation to obtain solid and liquid fractions. The analyses of these fractions from Composites A and B are given in Table II.

Upon repeated recrystallization in acetone, Composite A yielded three crops of acids weighing 26.5 gm. The mean molec-

ular weights of these fractions ranged from 255.1 to 255.8 and the melting points from 61.1–62.8°. These values indicated that normal palmitic acid predominated in Composite A. The solid acids of Composite B were converted to their methyl esters and distilled into three fractions: (a) mean molecular weight 270.9, (b) mean molecular weight 274.7, and (c) mean molecular weight 291.3. Crystallization of the acids obtained from fraction (a) yielded normal palmitic acid with a melting point of 62.5–62.8°; while fraction (c) yielded normal stearic acid with a melting point of 69.5–69.8°. The mother liquors from the crystallizations of palmitic acid from Composite A and fraction (a) of Composite B were combined and another lead salt-ether separation was made from which 4.2 gm. of solid acids were obtained. The melting point of these acids after several crystallizations was 56.5–57.0°, which is nearly that reported for isopalmitic acid (57.0–57.5°) by Yokoyama (4). The mean molecular weight, however, was found to be somewhat higher than theory (found 259.6, theory 256.3). The methyl esters were prepared and distilled through a modified Claisen flask and 2.6 gm. collected for the first fraction. The recrystallized fat acids obtained upon saponification melted at 61–61.5°. This melting point did not change upon further crystallization, and a mixed melting point with a sample of purified normal palmitic acid was obtained at 61.5–62.0°. The material crystallized in short needles, whereas normal palmitic acid under the same conditions formed lustrous scale-like flakes. It is probable that the low melting point and variation in crystalline structure were due to traces of other acids. The material remaining after distillation was recrystallized and the melting point and saponification value determined. Both determinations showed the material to be largely stearic acid. The evidence indicated that both normal palmitic and stearic acids were undoubtedly present and that little or no isopalmitic acid was present.

The bromination of liquid acids of Composites A and B and study of the resulting bromides showed the presence of the di- and tetrabromides of oleic and linoleic acids respectively.

The fat acids from Fractions 7, 8, and 9 (Table I) were separated by the lithium salt-acetone method. Fraction 7 yielded stearic, oleic, linoleic, and traces of a more highly unsaturated

acid; Fraction 8 yielded oleic, linoleic, and small amounts of clupanodonic acid which was identified by analysis of its brominated derivative (70.4 per cent bromine; theory for decabromobehenic acid, 70.8 per cent bromine). From Fraction 9 were obtained 14.2 gm. of acids whose lithium salts were soluble in acetone at 0–5°. The iodine number of these acids was 376.9 (theory for clupanodonic acid 384.5). After hydrogenation with Pd-BaSO₄ catalyst, the recrystallized saturated product melted at 81–81.5°. The saponification equivalent was found to be 337.0. By direct titration in neutral alcohol, the mean molecular weight was found to be 339.6. (Theory for the molecular weight of behenic acid is 340.3.)

The acids recovered from the residue (Fraction 10, Table I) were brominated. Insoluble bromides were obtained which were found to contain 71.3 per cent bromine. This fraction was considered as clupanodonic acid in the calculation of the fat composition.

Fat Acids of Egg Yolk Glycerides—From 556 gm. of purified glycerides, 519.5 gm. of mixed acids were obtained with an iodine number of 73.5 and a mean molecular weight of 275.3. A lead salt-alcohol separation yielded 172.0 gm. of solid acids (mean molecular weight 263.5, iodine number 7.38) and 347.5 gm. of liquid acids (mean molecular weight 278.9, iodine number 105.4). The methyl esters of the solid and liquid acids were prepared, methyl alcohol and dry HCl gas being used, and appropriate amounts were fractionally distilled. The distillation data and analyses of the distilled fractions representing all the solid acids and approximately 154 gm. of the liquid acids are given in Tables III and IV.

The saturated acids from Fractions 1 to 7 of Table III were separated by repeated crystallization. From the melting points and molecular weights, normal palmitic and stearic acids were identified. Traces of an acid of lower molecular weight could not be positively identified, but for purposes of calculation it was considered as myristic acid. Hexadecenoic acid was isolated and identified (8) from Fractions 1 and 2 of the liquid acids given in Table IV. The unsaturated linkage in this specimen was found to be in the 9,10 position (8). Fractions 4 to 9 proved to be methyl oleate and methyl linoleate. Redistillation of these

fractions produced no change in analyses and it was concluded from the constancy of the iodine numbers and mean molecular weights that the boiling points of these esters were too close to permit fractionation by distillation. Linoleic acid was identified by the characteristic tetrabromostearic acid, m. p. 113.5–114.0°.

The acids of Fractions 10 and 11 (Table IV) were found to be a mixture of oleic and linoleic acids and a highly unsaturated acid whose bromide was found to contain 69.7 per cent bromine. The bromide blackened but did not melt or contract in a closed tube up to 270°. Because the bromine content was intermediate to that of arachidonic and clupanodonic acids, it was decided to distil the rest of the methyl esters of the total mixed unsaturated

TABLE III

Yields and Characteristics of Fractions of Methyl Esters of Solid Fat Acids from Egg Yolk Glycerides, and Their Analyses

Fraction No.	Weight	B.p., 0.5–1.0 mm.	Mean mol. wt.	I No.
	gm.	°C.		
1	31.95	125–130	268.6	0.30
2	34.06	130	269.5	0.30
3	48.33	130	270.8	0.80
4	8.34	130–140	279.5	3.10
5	25.47	140	290.8	14.20
6	28.00	140–145	297.8	28.30
7	4.12	Residue	299.0	23.60

acids in order to secure an additional concentrated fraction of the acid or acids in question. A fraction comparable to Fraction 11 was obtained, which, combined with the remainder of Fraction 11 (Table IV), was redistilled. About 3 gm. of methyl esters having an iodine number of 283.6 and a mean molecular weight of 326.3 were obtained. This fraction was hydrogenated with Pd-BaSO₄ as catalyst. The saturated esters were recrystallized several times from methyl alcohol and melted at 51.2–52.0°. The acids obtained from the hydrogenated ester, after six crystallizations from acetone, melted at 79.8–80.2°. A mixed melting point with the behenic acid fraction obtained from lecithin (m. p. 81.0–81.5°) melted at 79.8–80.4°. The specimen from the glyce-

erides was apparently less pure than that from lecithin but can be considered as essentially the same; namely, the hydrogenated

TABLE IV
Yield and Characteristics of Fractions of Methyl Esters of Liquid Fat Acids from Egg Yolk Glycerides

Fraction No.	Weight	B.p., 0.5-1.0 mm.	Mean mol. wt.	I No.
	<i>gm.</i>	<i>°C.</i>		
1	4.45	125-135	264.3	61.22
2	13.08	135-137	277.7	67.40
3	25.00	137-141	294.5	91.10
4	25.70	141-143	295.9	98.20
5	26.05	143	296.1	98.90
6	24.10	143	296.3	98.40
7	20.55	143	295.9	98.20
8	3.81	143	296.4	98.30
9	1.95	143	296.7	98.50
10	1.32	143-145	299.0	111.10
11	4.79	145-158	320.6	223.40
12	2.93*	Residue		

* The residue had a dark red color.

TABLE V
Component Fat Acids in Lecithins and Glycerides of Egg Yolk

Acid	Lecithin fat acids		Glyceride fat acids	
	Per cent by weight	Molecular per cent	Per cent by weight	Molecular per cent
Myristic.....			0.7	0.8
Palmitic.....	31.8	34.6	25.2	27.0
Stearic.....	4.1	4.0	7.5	7.3
Palmitoleic.....			3.3	3.6
Oleic.....	42.6	42.0	52.4	51.0
Linoleic.....	8.2	8.2	8.6	8.4
Clupanodonic.....	13.3	11.2	2.3	1.9
Total saturated.....	35.9	38.6	33.4	35.13
“ unsaturated.....	64.1	61.4	66.6	64.87

product of clupanodonic acid. The possibility of these fractions being mixtures of two or more of C₂₀, C₂₂, C₂₄, or C₂₆ acids, however, cannot be entirely eliminated.

Calculation of Fat Acid Composition—The percentage composition and the molecular percentage composition of each acid in lecithin fat acids and glyceride fat acids have been computed, the results of which are given in Table V.

DISCUSSION

From a qualitative standpoint, the results agree in most respects with the findings of Levene and Rolf (2), with the exception that in the present work the evidence was indicative of the presence of clupanodonic acid rather than of arachidonic acid. It is possible that this difference may be due to dietary differences. Cruickshank (9) has shown that the iodine numbers of the mixed acids of egg yolk can vary from 80.0 to 127.2, depending on the nature of the dietary fat. The identification of clupanodonic acid by Yokoyama (4) supports the present findings. The present data on the saturated acids of lecithin suggest that the reports of the occurrence of isopalmitic acid (4) and of margaric acid (3) may have been in error through mistake in identification as a mixture of palmitic and stearic acids.

It is of interest to note that hexadecenoic acid was found in appreciable amounts in the glyceride fraction but none was detected in the lecithin fraction. The body fat of the hen was found by Hilditch, Jones, and Rhead (10) to contain from 6 to 7 per cent palmitoleic acid. Thus there is apparently a distinction between lecithin and glycerides as regards the presence in appreciable amounts of at least one acid. The low yield of cephalin is in harmony with that reported by Nishimoto (11) and at variance with some of the older reports.

SUMMARY

Lecithin and glyceride fractions of high purity were prepared from egg yolk, in sufficient quantities to make a study of the fat acid composition.

Palmitic and stearic acids were the only saturated acids identified. Some evidence of small amounts of a lower saturated acid was found in the glyceride fraction.

Oleic, linoleic, and clupanodonic acids were found in lecithins and glycerides, and in addition, 9,10-hexadecenoic acid was isolated from the latter.

The percentage composition of the individual acids reported represents a reasonable approximation of the fat acid composition of the glyceride and lecithin fractions of egg yolk lipids obtained from eggs produced on a representative egg-laying ration.

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SOME CHARACTERISTICS OF THE ANDROGENIC FRACTIONS FROM BULL URINE*

BY LEWIS W. BUTZ AND S. R. HALL

*(From the Bureau of Animal Industry and the Bureau of Dairy Industry,
United States Department of Agriculture, Beltsville, Maryland)*

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The presence of androgens in bull urine is now established (1, 2). The apparent daily excretion, as measured by the methods in use for the hydrolysis, extraction, and assay of human urines, is much smaller than that of men and women. Indeed some healthy mature bulls do not appear to excrete any androgen ((3) and our unpublished observations). It is possible then that bulls excrete very little androgen or that they excrete appreciable amounts of active substances which are less stable than androsterone and dehydroisoandrosterone¹ and consequently cannot withstand the vicissitudes of the hydrolysis and extraction. It is also conceivable that the androgens of bull urine are obtained in a form which is unsuitable for assay by the smear method. Admixed² or conjugated³ substances may partially inhibit the comb growth-promoting effect. The androgenic and other steroids of the urine of cattle are of particular interest because only from this species have androgens from the testes (5) and adrenal glands (6) been identified.

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¹ Dehydroisoandrosterone gives an inactive chloroandrostenone with HCl, and this same chloro compound has been isolated from human urine after treatment with HCl (4).

² The proportion of inactive substances in the neutral fraction of the benzene extract from bull urine is about 1000 times greater than that in some extracts we have made from the urine of men.

³ Some esters have a delayed or reduced action when applied to the comb directly. We have had to use the smear assay because the quantities of material available have been insufficient for assay by intramuscular injection.

In the present paper some experiments will be described which indicate that at least a part of the androgens of bull urine are different from those (7) which have been isolated from human urine. It is probable but not proved that this part consists of α,β -unsaturated ketones. Since we shall not have, for some time to come, enough material for identification of the androgenic components, it seems best to report our preliminary findings now.

Only the neutral fraction of the benzene extract has been examined. All of the activity of this fraction is associated with the ketones and these are partially inactivated by heating with KOH in methanol. The ketones were fractionated by the procedure of Anchel and Schoenheimer (8) by which these investigators realized an excellent separation of cholestanone and cholestenone. When applied to the ketones of our extract of urine, this method effected a separation into two fractions, approximately equal in weight. Most of the androgenic activity was associated with the "cholestenone" fraction. This fraction exhibited an absorption spectrum⁴ characteristic for α,β -unsaturated ketones (9). We studied also the behavior of testosterone acetate toward the Anchel and Schoenheimer reagents. Under the conditions used most of the ester was recovered in the "cholestenone" fraction, but some of the hydrazone was split by formaldehyde. 1 liter of bull urine yielded 6 mg. of cholestenone fraction. The assays indicate that this fraction cannot contain more than 0.01 per cent of androgen if the androgen has a potency of the order of that of androsterone and not more than 1.0 per cent of androgen if the androgen of this fraction is relatively weak such as the androsta-dienone isolated by Burrows *et al.* (10).

EXPERIMENTAL

The material used in this work was obtained from the urine of an 8 year-old Jersey bull. Collections were made during the period, June to October, 1937. The procedure for hydrolysis and extraction of the urine was that previously (2) employed. The urine was refluxed with the acid for 1 hour prior to extraction.

⁴ We are indebted to Mr. N. R. Ellis and Dr. Russell E. Davis, Animal Nutrition Division, Bureau of Animal Industry, Beltsville, for these measurements. The cholestenone used for comparison was prepared by Dr. Eleanore W. J. Butz to whom we wish to express our thanks.

Benzene was not added during the refluxing.⁵ The neutral part of the benzene extract from 770 liters of urine weighed 106 gm. This neutral fraction had a potency, when assayed by direct application to the comb of capons, of 0.01 microgram per mg. (as androsterone). The ketones, separated from 17 gm. of this fraction by Girard and Sandulesco's Reagent T (12), had a potency of 0.06 microgram per mg. Since the yield of ketones was 2.53 gm., substantially all the activity was found in the ketone fraction. Removal of ketones was considered to be complete, as no semicarbazone was isolated from the residue.

Alkaline Hydrolysis of Neutral Fraction—22 gm. of the neutral fraction were refluxed for 2 hours with 45 cc. of 4 N KOH in methanol. Only 11.09 gm. of unsaponifiable material and 2.80 gm. of acids were recovered. The large amount of material lost probably consisted of water-soluble and volatile substances, since no measures for the recovery of these were taken.

From 9 gm. of this unsaponifiable material, 1.29 gm. of ketones were separated by Reagent T. This ketone fraction had a potency of 50 micrograms per gm. (as androsterone).

The total androgen recoverable from the 22 gm. of neutral fraction after saponification is therefore equivalent to 79 micrograms of androsterone. Assay of the whole neutral fraction showed a content of 220 micrograms in 22 gm. and the concentration calculated from the assays of the "cholestanone" and "cholestenone" fractions is 198 micrograms per 22 gm. During saponification, then, 60 to 64 per cent of the androgenic activity was destroyed.

Reaction of Urine Ketones with p-Carboxyphenylhydrazine—2.53 gm. of ketones were refluxed for 4 hours in 40 cc. of 95 per cent ethanol containing 15 drops of acetic acid with 7 gm. of *p*-carboxyphenylhydrazine. 4 gm. of dry hydrazones were obtained, corresponding to an average molecular weight of the ketones of 312 if 80 per cent recovery of the hydrazones is assumed. The hydrazones were refluxed for 6½ hours in 70 cc. of 95 per cent ethanol with 8 cc. of 37 per cent aqueous formaldehyde. The weight of liberated ketones ("cholestanone" fraction) was 1.32 gm. The

⁵ It has recently (11) been reported that in the case of human urine when benzene is present during the hydrolysis with acid the destruction of androgen is reduced.

undecomposed hydrazones were then refluxed for 4 hours in 60 cc. of 95 per cent ethanol with 12 cc. of pyruvic acid; 1.06 gm. of "cholestenone" fraction were thus obtained.

Reaction of Testosterone Acetate with p-Carboxyphenylhydrazine—500 mg. of the ester were refluxed in 20 cc. of 95 per cent ethanol for 2 hours with 1 gm. of reagent. A 70 per cent yield of hydrazone, m.p. 260° with decomposition, was obtained. No purification of this product other than drying over P_2O_5 *in vacuo* was attempted. 430 mg. of the hydrazone were refluxed in 17 cc. of 95 per cent ethanol containing 0.3 cc. of 37 per cent formaldehyde for 6 hours. This treatment split a part of the hydrazone; 365 mg. of hydrazones were recovered from the K_2CO_3 solution and 61 mg. of liberated ketone from the ether layer. About 20 per cent was split by formaldehyde. The undecomposed hydrazones in 20 cc. of 95 per cent ethanol were refluxed for 4 hours with 2 cc. of pyruvic acid. This liberated 60 per cent more of the testosterone acetate. 16 per cent was recovered as *p*-carboxyphenylhydrazone which was not decomposed by the pyruvic acid.

Bioassay Procedure and Data—White Leghorn capons, 8 months old or older, were used for the assays. The urine fractions were dissolved in tricaproin and 0.125 cc. of solution was smeared on the comb daily for 5 days. Androsterone in tricaproin was given to a control group of birds. A preliminary assay of "cholestenone" and "cholestanone" fractions, from another batch of ketones than that described above, at a 3 mg. daily dose gave a threshold response in the case of the "cholestenone" fraction and no growth with the "cholestanone" fraction. In the confirmatory experiment 9 mg. of cholestenone fraction, 15 mg. of cholestanone fraction, and 30 mg. of the ketones, separated after saponification, were given during the same week. The growth responses (Table I) observed were equivalent to those following 1 microgram, less than 0.5 microgram, and 1.5 micrograms of androsterone respectively.

A number of whole neutral extracts prepared from the urines of various bulls have also been administered to capons by intramuscular injection. 1 cc. of the tricaproin solution daily was given by this route. The ratio of potency by the smear technique to potency by intramuscular injection is of the order of 10. The ratio for androsterone as determined under our conditions is 200.

Although too few experiments have been carried out to permit attributing a high degree of accuracy to the former value, it can be stated that the composite androgens of the bull urine exhibit a much lower ratio than does androsterone. Voss (13) has determined this ratio for a number of pure compounds. He found testosterone to have a ratio of between 5 and 10, which was lower than that of any of the compounds examined. Androsterone in his hands had a ratio of 35.

TABLE I
Bioassay of Urine Fractions by Comb Growth

The solution of the fractions was smeared on the comb daily for 5 days.

No. of capons	Material	Comb increase, length + height*
		mm.
8	Androsterone	8.0 (4, 15)
8	"	10.6 (8, 15)
7	"	15.4 (10, 18)
8	"	18.6 (10, 30)
7	"	7.0 (5, 13)
7	"	11.8 (8, 16)
10	"	6.8 (2, 12)
		mg. per day
4	Neutral extract†	5.0 (3, 9)
4	Anones (total)	4.0 (2, 5)
7	Enones "	10.7 (9, 13)
4	Ketones (total), after saponification	12.5 (11, 14)
4	Acids (total)	0.0 (0, 1)

* The values given are averages. The minimum and maximum responses are shown in parentheses.

† Given on 4 days only.

DISCUSSION

All of the evidence suggests that the androgens excreted in the urine by the bull are α, β -unsaturated ketones or substances with very similar properties. The instability toward alkali recalls the experience of others with extracts of bull testicle (14) and with pure testosterone (5), and emphasizes again the inadvisability of employing alkaline hydrolysis during the isolation of natural androgens.

It would be desirable to know how all the androgenic chemical types behave when their *p*-carboxyphenylhydrazones are successively treated with formaldehyde and pyruvic acid. On the basis of the behavior we have observed for testosterone acetate, it seems possible that the small activity of the "cholestanone" fraction could be due to contamination with α,β -enones. Unfortunately the spectrographic evidence does not facilitate a decision of this point, for while the "cholestenone" fraction exhibited a strong band with a characteristic maximum at 2380 Å., the "cholestanone" fraction showed continuous absorption throughout this region and into the shorter wave-lengths without a maximum.

The probable occurrence of androgens with olefin linkages adjacent to other functional groups in the urine of bulls makes it imperative to develop milder methods of hydrolysis and extraction. It is possible that with the help of such methods higher levels of androgen excretion will be found not only for the bull but for other species as well. Also the isolation of new excretion products can be expected.

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THE EFFECT OF PYRROLE ON THE OXIDATION OF AMINES AND THE NON-NATURAL ISOMERS OF CERTAIN AMINO ACIDS

BY FREDERICK BERNHEIM, MARY L. C. BERNHEIM, AND
HARRY O. MICHEL

*(From the Departments of Physiology and Pharmacology and Biochemistry,
Duke University School of Medicine, Durham, North Carolina)*

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It was shown some time ago that pyrrole could act catalytically on the oxidation of lactic acid by rat liver (1). The effect was small and did not occur if unwashed tissue was used or if the tissue was washed completely free of hemoglobin. These facts suggested that pyrrole replaced a catalytic system removed by the washing but that hemoglobin was necessary for the reaction to occur. We have reinvestigated the effect of pyrrole and have been able to confirm our previous findings and to extend them to show that both pyrrole alone and pyrrole with hemoglobin act as powerful catalysts for the oxidation of amines and certain non-natural amino acids, and that pyrrole catalyzes the formation of methemoglobin from hemoglobin by tissues.

EXPERIMENTAL

Effect of Pyrrole on Oxidation of Amines—Because we were studying the acceleration of the oxidation of amines, we chose rat liver as the source of the amine oxidase, for this oxidizes amines more slowly than the guinea pig or rabbit liver. Approximately 4 gm. of rat liver were chopped with scissors, ground in a mortar with sand and 10 cc. of 0.05 M phosphate buffer of pH 6.7, and squeezed through muslin. 0.5 cc. of the resulting suspension was put in a Warburg vessel and the volume made up to 2.0 cc. with substrate and buffer. Various amines were added with and without 1.0 mg. of pyrrole, which had been freshly distilled. The final pyrrole concentration was M/134. The results are shown in

Table I. The greatest acceleration was with β -phenylethylamine as substrate, though all the other amines showed some effect except mescaline. This confirms our previous finding that the oxidation of mescaline proceeds by a somewhat different mechanism from the oxidation of the other amines (2). Isoamylamine is oxidized more rapidly and to a greater extent by the enzyme alone than the other amines and the percentage acceleration by pyrrole is less. This is important because the extra oxygen uptake in the presence of pyrrole might be caused by its secondary oxidation by the H_2O_2 which is a by-product of the amine oxidation (3). If

TABLE I

Effect of 1.0 Mg. of Pyrrole on Oxygen Uptake of 1.0 Mg. Each of Various Amines with Untreated Rat Liver Suspension, pH 6.7 at 37°

The values are given in c.mm.

Time		β -Phenyl-ethylamine hydrochloride		β -Oxy- β -phenylethylamine hydrochloride		Isoamylamine hydrochloride		Tyramine		Mescaline sulfate	
		Alone	Pyrrole	Alone	Pyrrole	Alone	Pyrrole	Alone	Pyrrole	Alone	Pyrrole
<i>hrs.</i>	<i>min.</i>										
	15	14	36	8	7	26	16	13	17	7	2
	25	15	104	13	20	44	58	10	55	9	3
	50	17	196	14	48	57	127	11	88	2	4
1	5	19	235	15	72	67	151	9	90	2	15
1	55	15	305	16	112	79	199	2	82	6	16
2	25	23	329	18	125	79	198	-8	61	-2	23
2	55	24	331	17	136	74	196	-20	50	-5	25
3	20	25	329	17	141						

this were so, a greater percentage acceleration of the oxygen uptake would be expected when isoamylamine was being oxidized. Since this is not the case, it indicates that pyrrole is acting as a catalyst and that the extra oxygen uptake in its presence is not accounted for by its own oxidation. The pyrrole and amine concentration curves described below confirm this, as does the fact that the pyrrole effect is less in guinea pig and rabbit liver where the oxidation of the amine itself and consequently the production of H_2O_2 proceeds to a greater extent.

Preparation of Enzyme—It was necessary to determine whether

hemoglobin or methemoglobin played any part in the reaction. For this purpose a standard preparation was used with β -phenylethylamine. The liver suspension was diluted with water to 50 cc. and centrifuged. The liquid was removed and the precipitate resuspended in water to which 5 to 10 cc. of buffer of pH 6.7 were added. This process was repeated four times and the precipitate finally suspended in 10 cc. of buffer. The liquid from the fourth washing contained from 1 to 2×10^{-6} M hemoglobin; so in order to see whether the pyrrole effect was dependent on the presence of hemoglobin a preparation was washed six more times, making a total of ten washings. After the sixth no more hemoglobin could be detected in the washings by the spectrophotometric method used, of which the limit with 6.0 cm. was 5×10^{-7} M. When pyrrole was added to liver washed either four or ten times, the acceleration was still marked but the uptake stopped when 2 atoms of oxygen had been taken up. This indicated that pyrrole could act catalytically in the absence of hemoglobin. But when hemoglobin or methemoglobin was added in a concentration of 2×10^{-5} M, the oxygen uptake proceeded more rapidly and went on until 4 atoms of oxygen had been taken up (Fig. 1, A and B). Because hemoglobin was always rapidly converted to methemoglobin, the latter was used in these experiments. Washed human cells were laked with toluene, centrifuged, ferricyanide was added, and the mixture was dialyzed for 24 hours against running water. The methemoglobin was then diluted so that the final concentration in the Warburg vessel was 1 to 3×10^{-5} M, which was the optimum for the acceleration. Dog methemoglobin twice recrystallized behaved exactly like the human pigment. Methemoglobin without pyrrole accelerated the oxidation only very slightly. It was shown that the oxidation of the various amines was accelerated in the same relative way by the standard preparation with pyrrole and methemoglobin as by the crude liver preparation.

Effect of Amine and Pyrrole Concentration—Fig. 1, A and B, shows the action of pyrrole and pyrrole + methemoglobin on the oxidation of different concentrations of β -phenylethylamine. The extra oxygen uptake in both cases was a function of the concentration of amine, indicating that pyrrole is acting as a catalyst. This is also indicated by Fig. 2 which shows that with widely different

concentrations of pyrrole the final uptake was not a function of the amount of pyrrole present. Although relatively large concentrations of pyrrole are necessary to obtain the acceleration, the evidence indicates that it is acting as a catalyst and not as a substrate in the reaction.

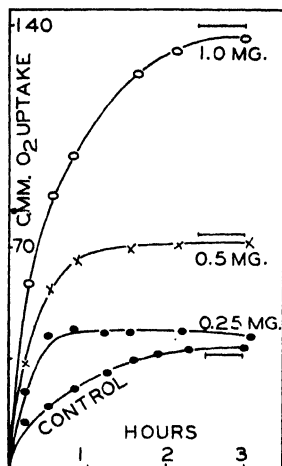


FIG. 1, A

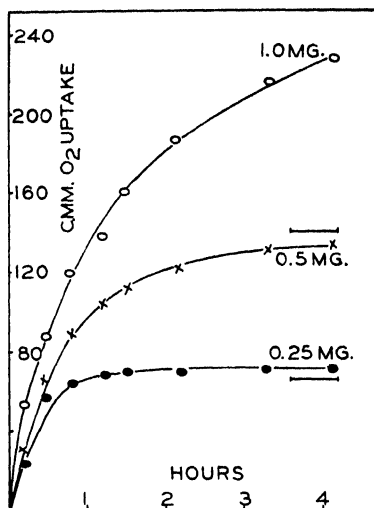


FIG. 1, B

FIG. 1. The oxygen uptake of different amounts of β -phenylethylamine hydrochloride in the presence of the standard liver preparation plus 1.0 mg. of pyrrole (A), or plus 1.0 mg. of pyrrole with 2×10^{-5} M methemoglobin (B). The control in A represents the oxidation of 0.5 mg. of β -phenylethylamine hydrochloride by the preparation alone. pH 6.7 at 37°. In A the horizontal lines represent the theoretical uptake for 2 atoms per molecule; in B for 4 atoms per molecule of β -phenylethylamine hydrochloride.

That pyrrole is present at the end of the reaction is shown by the following test. The 2.0 cc. of reaction mixture were removed from the Warburg vessel and the protein precipitated by 2.0 cc. of 5 per cent trichloroacetic acid. The clear filtrate was warmed to boiling and 1 drop of Millon's reagent was carefully added without mixing. A copious precipitate formed at the bottom and an orange color formed in the clear solution above. This color rapidly changes on standing to red and then purple and it is pro-

portional to the concentration of pyrrole in a range of 0.25 to 4.0 mg. Comparative tests showed that the same amount of pyrrole was present in the control as in the vessels in which the pyrrole and pyrrole + methemoglobin accelerations had occurred.

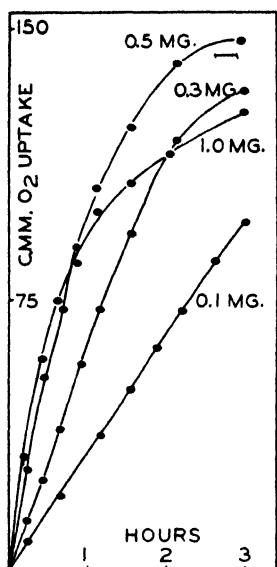


FIG. 2

FIG. 2. The effect of different amounts of pyrrole on the oxygen uptake of 1.0 mg. of β -phenylethylamine hydrochloride with the standard liver preparation. pH 6.7 at 37°. Similar curves are obtained when methemoglobin is added to the pyrrole. The horizontal line represents the theoretical uptake for 2 atoms per molecule of β -phenylethylamine hydrochloride.

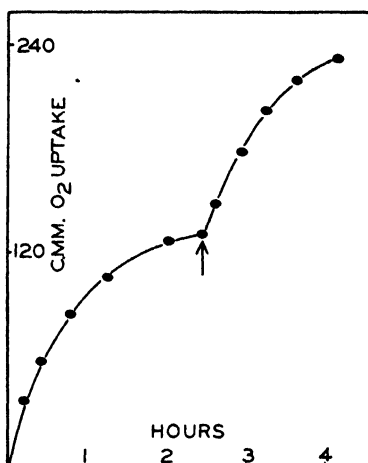


FIG. 3

FIG. 3. The oxygen uptake of 1.0 mg. of β -phenylethylamine hydrochloride in the presence of the standard liver preparation and 1.0 mg. of pyrrole. 2×10^{-5} M methemoglobin was added at the arrow. pH 6.7 at 37°.

The color is not given by pyridine or proline. Details of the test will be described elsewhere.

Properties of Pyrrole and Pyrrole Plus Methemoglobin Accelerations—Fig. 3 shows the effect of methemoglobin added after the acceleration by the pyrrole alone was finished. This experiment showed that the methemoglobin acceleration is not dependent on

the simultaneous acceleration by the pyrrole and therefore the effect of the methemoglobin is not caused by a coupled oxidation. If the mixture was boiled for 3 minutes after the pyrrole effect was finished and before the methemoglobin was added, then the methemoglobin did not cause any further oxidation. This showed that the oxidation is dependent on a thermolabile catalyst in the liver preparation and that the methemoglobin cannot catalyze the reaction alone. During the oxidation of the amine by the pyrrole + methemoglobin enzyme mixture, a small amount of the methemoglobin disappeared. In a typical experiment the methemoglobin concentration was 2.0×10^{-5} M in the control and 1.3×10^{-5} M after the oxidation.

The tissue showed no increase in activity after incubation at room temperature with pyrrole and subsequent washing. If a protein-pyrrole complex is formed, it must readily dissociate.

Effect of Inhibitors—0.002 M KCN and 0.5 per cent potassium pyrophosphate inhibited completely the acceleration by pyrrole and by pyrrole + methemoglobin, although the slow oxidation of the amine by the enzyme alone was unaffected by either drug. 2 per cent sodium fluoride had no effect on the pyrrole acceleration but inhibited 40 to 60 per cent of the pyrrole + methemoglobin effect. This is probably due to the formation of fluorine-methemoglobin. This is further evidence that the pyrrole effect is not caused by traces of hemoglobin remaining in the liver preparation and that it is therefore distinct from the pyrrole + methemoglobin effect. 1 per cent urethane had no effect on either system. Both accelerations were slightly less at pH 7.8 than at pH 6.7.

Effect of Oxygen Tension—Kohn (4) has shown that the rate of oxidation of the amines can be increased by increasing the oxygen tension. It was of interest therefore to test the effect of increased oxygen tension on the pyrrole acceleration. The results are shown in Table II. The pyrrole, methemoglobin, and the pyrrole + methemoglobin accelerations were increased by the increased oxygen tension.

Specificity of Pyrrole Effect—N-Methyl pyrrole and N-ethyl pyrrole accelerated the amine oxidation, but less than pyrrole itself. No C-substituted pyrroles have yet been tried. Pyrrole which had been reduced to a mixture of pyrroline and pyrrolidine by platinum and hydrogen (the reduction being checked by the

increased alkalinity of the solution) behaved like pyrrole. Pyridine was without effect in a large concentration range. Methylene blue cannot be substituted for methemoglobin.

Pyrrole or pyrrole + methemoglobin has no effect on the oxidation of acetate, glucose, pyruvate, succinate, choline, histamine, or the natural isomers of the amino acids. The oxidation of ethyl alcohol was inhibited 40 per cent. In the standard preparation there was no effect on the oxidation of xanthine and hypoxanthine, though in the unwashed liver suspension a slight acceleration was sometimes seen. The accelerating effect on lactate has

TABLE II

Effect of Oxygen Tension on Oxidation of 1.0 Mg. of β -Phenylethylamine Alone and with 1.0 Mg. of Pyrrole, 2×10^{-5} M Methemoglobin, and the Two Together, pH 6.7 at 37°

The values are given in c.mm.

Time		Air				100 per cent oxygen			
		Alone	Methemoglobin	Pyrrole	Pyrrole + methemoglobin	Alone	Methemoglobin	Pyrrole	Pyrrole + methemoglobin
hrs.	min.								
	15	8	8	13	22	22	50	29	57
	35	12	20	45	79	41	77	86	158
	50	16	28	64	122	58	101	113	214
1	15	18	33	80	158	77	128	135	253
1	45	27	40	93	186	96	156	153	278
2	10	31	47	103	206	119	177	162	288

already been mentioned. The oxidation of the non-natural isomers of the amino acids is, however, definitely accelerated by pyrrole and by pyrrole + methemoglobin. This is particularly true for alanine.

Effect of Pyrrole on Oxidation of Non-Natural Isomers of Amino Acids—The non-natural isomer of alanine was oxidized very slowly by the standard liver preparation but more rapidly by rat kidney prepared in the same way as the liver. In both cases pyrrole and pyrrole + methemoglobin caused a marked acceleration. In order to compare the effect on the alanine with that on the non-natural isomers of other amino acids the rat kidney preparation was used.

Table III shows the accelerating effect of the pyrrole and pyrrole + methemoglobin on the oxidation of several amino acids. The methemoglobin alone had no effect. The accelerating effect was most marked for alanine and, as in the case of β -phenylethylamine, the oxygen uptake is a function of the alanine concentration. The end-points, however, do not correspond to the uptake of any definite number of atoms of oxygen per molecule. In the presence of pyrrole the uptake usually stopped when between 1 and 2 atoms of oxygen had been utilized and with pyrrole + methemoglobin

TABLE III

Oxygen Uptake of Non-Natural Isomers of Certain Amino Acids (1.0 Mg. Each) with Washed Rat Kidney Preparation

The effect of 1.0 mg. of pyrrole and 1.0 mg. of pyrrole + 2×10^{-5} M methemoglobin, pH 6.7 at 37°. For each amino acid, Column 1 represents enzyme alone; Column 2, enzyme + pyrrole; Column 3, enzyme + pyrrole + methemoglobin. The figures are c.mm. of oxygen.

Time		Alanine			Valine			Methionine			Proline			Leucine			Serine		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
hrs.	min.																		
	15	16	28	51	8	10	37	7	18	23	30	27	24	4	5	4	2	5	6
	35	36	57	96	20	28	72	10	30	45	45	45	50	4	7	7	3	6	10
	55	48	86	132	26	44	102	21	52	78	64	66	79	7	17	21	2	10	20
1	25	75	123	160	43	62	122	26	74	101	67	74	88	8	21	29	4	17	30
1	45	80	126	174	44	68	129	31	90	112	69	90	103	2	34	35	5	20	33
2	35	91	148	196	47	79	143				73	96	109	2	39	53			
3	50	101	163	213	46	80	191												
4	45	117	165	220	47	80	197												

it stopped between 3 and 4 atoms. The pyrrole concentration curve is similar to that for β -phenylethylamine. Increased oxygen tension did not increase the acceleration, showing that this is not a property of the pyrrole system but is specific for the amine oxidase. KCN and pyrophosphate inhibited the acceleration but did not affect the oxidation of alanine by the enzyme alone.

Formation of Methemoglobin by Pyrrole—If pyrrole and hemoglobin were added to an unwashed tissue suspension made by grinding the tissue with sand and squeezing through muslin, the rate of methemoglobin formation was greatly accelerated. Pyridine or nicotinic acid had no such effect. For every 100 c. mm.

of oxygen uptake the kidney produced most extra methemoglobin, the liver next, and the brain produced no extra in the presence of pyrrole. Pyrrole shaken with hemoglobin alone under the same conditions does not form methemoglobin. It has been shown by Bernheim and Michel (5) that for every 100 c. mm. of oxygen uptake the kidney produces most methemoglobin, the liver next, and then the brain. The extra methemoglobin formation by pyrrole follows this order and this indicates that pyrrole is catalyzing systems already active in the tissues, for the methemoglobin production by tissues is a function of the oxygen uptake of the tissue and can be increased by the addition of substrates which form hydrogen peroxide, such as the amines and the non-natural isomers of the amino acids. The extra methemoglobin formation by the pyrrole could be explained on the basis of its accelerating action on the oxidation of amines and other substances that may be present in the tissue suspension. That tissue oxidation is accelerated by pyrrole is indicated by the fact that the oxygen uptake of the liver and kidney suspensions is increased by pyrrole. In the standard washed preparation of liver or kidney methemoglobin is rapidly formed from hemoglobin when an amine or an amino acid is being oxidized but is not formed in their absence, because the preparation itself takes up no oxygen.

Fig. 4 shows that the amount of extra methemoglobin formation is a function of the amount of liver present. The liver was in 0.05 M phosphate buffer of pH 6.7. The extra methemoglobin formation is proportional to the amount of pyrrole present in concentration ranges from 0.05 to 0.5 mg. of pyrrole. It increases with time and the rate of production parallels, except at the very beginning, the rate of oxygen uptake by the tissue (Fig. 5). This indicates the pyrrole effect is dependent on the presence of oxidizable substrates. Cyanide inhibits the extra methemoglobin production with pyrrole.

Products of Reaction. β -Phenylethylamine—When the amine was oxidized in the presence of pyrrole, exactly 2 atoms of oxygen were taken up, suggesting that phenylacetic acid was formed. The addition of methemoglobin caused another 2 atoms to be taken up, indicating that in its presence the acid was oxidized further. Addition of phenylacetic acid showed that it was not attacked by the enzyme + pyrrole + methemoglobin system. It was pos-

sible that the simultaneous oxidation of the amine was necessary for the oxidation of the acid but this was not the case. Attempts were made to isolate phenylacetic acid in a large scale experiment in which 150 mg. of the amine were oxidized in the presence of pyrrole. A yellow oil was obtained which gave an acid reaction but did not crystallize. Control experiments showed that added phenylacetic acid could be recovered almost quantitatively under

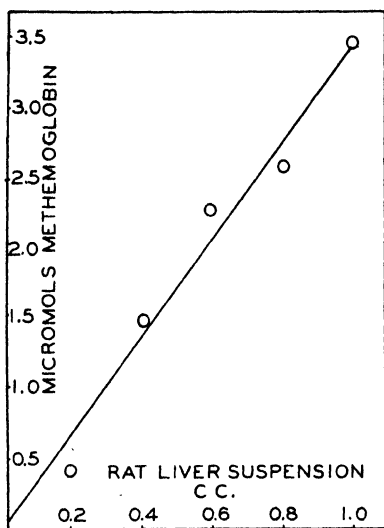


FIG. 4

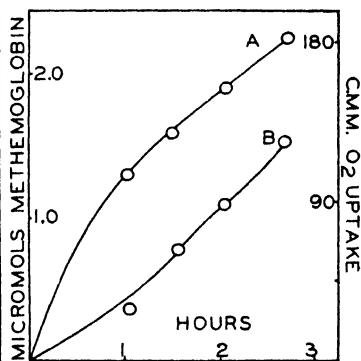


FIG. 5

FIG. 4. The effect of liver concentration on the extra methemoglobin produced from hemoglobin by 0.3 mg. of pyrrole, pH 6.7 at 37°.

FIG. 5. Curve A, the oxygen uptake of liver suspension corrected for methemoglobin formation; Curve B, the simultaneous production of methemoglobin by 0.2 mg. of pyrrole. pH 6.7 at 37°.

the same conditions and that no impurity in the preparation interfered with the crystallization. An oil with a deep brown color was obtained when the amine was oxidized in the presence of pyrrole and methemoglobin. Neither oil gave positive tests with ferric chloride or Millon's reagent. Ammonia was produced during the oxidation with pyrrole and pyrrole + methemoglobin. A small amount of decarboxylation occurred in both cases, but the amount of CO₂ produced would account for the decarboxylation of

less than 10 per cent of the substance present. No bisulfite-binding substances were formed.

Alanine—Alanine was deaminated. As in the case of β -phenylethylamine, decarboxylation occurred to the extent of about 10 per cent of the theoretical in the presence of pyrrole and pyrrole + methemoglobin. The production of pyruvic acid was estimated by the method of Clift and Cook (6). After the oxidation of the alanine by the enzyme alone 56 per cent of the theoretical was recovered; in the presence of pyrrole, 26 per cent; in the presence of pyrrole and methemoglobin, 14 per cent. Controls with pyruvic acid gave the following results. Pyruvate shaken alone at pH 6.7 gave a 92 per cent recovery, with methemoglobin 83 per cent, with pyrrole 96 per cent, with kidney 49 per cent, with kidney and methemoglobin 29 per cent, with kidney and pyrrole 47 per cent, with kidney, methemoglobin, and pyrrole 27 per cent. As in the case of the β -phenylethylamine, the end-products were not what might be theoretically expected from the oxygen uptake, but the fact that decarboxylation occurred in both cases indicates that other reactions are taking place.

DISCUSSION

Because pyrrole accelerates the oxygen uptake in the presence of substrates which produce hydrogen peroxide, there was the possibility that the extra oxygen uptake could be accounted for by the secondary oxidation of pyrrole by the hydrogen peroxide formed by the primary oxidation of the amine or non-natural isomers of the amino acids. This, however, cannot be the case because (a) the more actively the amine is oxidized by the enzyme alone the smaller is the percentage acceleration by the pyrrole; (b) the extra oxygen uptake caused by the pyrrole is a function only of the amine or amino acid concentration and not of the pyrrole concentration within limits; and (c) the effect is very specific, for N-methyl and N-ethyl pyrrole are much less effective and pyridine and proline have no accelerating action.

It appears that pyrrole forms with the protein a catalytically active system for the oxidation of amines and non-natural isomers of the amino acids and that this system may react with methemoglobin so that the oxidation may be carried further.

SUMMARY

1. Pyrrole in concentrations varying from $M/1340$ to $M/134$ accelerates the oxidation of amines by washed rat liver preparation, particularly β -phenylethylamine which takes up 2 atoms of oxygen per molecule in the presence of pyrrole.

2. The addition of $2 \times 10^{-5} M$ methemoglobin further increases the acceleration and 4 atoms of oxygen are taken up per molecule of β -phenylethylamine. Methemoglobin alone has only a very slight effect.

3. In guinea pig and rabbit livers, which oxidize the amines more rapidly, pyrrole and pyrrole + methemoglobin have correspondingly less effect.

4. KCN and pyrophosphate completely inhibit both the pyrrole and the pyrrole + methemoglobin acceleration. Fluoride has no effect on the pyrrole but inhibits 40 to 60 per cent of the pyrrole + methemoglobin acceleration.

5. Increased oxygen tension increases the accelerating effect of pyrrole, methemoglobin, and pyrrole + methemoglobin as well as increases the oxidation of the amines by the enzyme alone.

6. The oxidation of certain non-natural isomers of the amino acids, particularly alanine by washed rat kidney preparation, is similarly accelerated by pyrrole and pyrrole + methemoglobin. No definite number of atoms of oxygen is taken up per molecule of alanine oxidized. Increased oxygen tension has no effect.

7. N-Methyl and N-ethyl pyrrole have an effect similar to pyrrole but they are less active. Pyridine is inactive.

8. Except for a slight acceleration of the oxidation of lactate and a slight effect on the oxidation of hypoxanthine, pyrrole and pyrrole + methemoglobin do not accelerate the oxidation of any of the other substrates tried.

9. The formation of methemoglobin by kidney and liver suspensions is greatly increased by pyrrole. Kidney shows a greater effect than liver but brain shows no extra methemoglobin formation. The extra methemoglobin formed is proportional to the concentration of the tissue suspensions, varies within limits with the pyrrole concentration, and parallels the oxygen uptake of the tissue.

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THE OXYGEN UPTAKE OF TISSUES IN VITAMIN DEFICIENCIES*

BY BARNETT SURE AND JAMES B. DEWITT

(From the Department of Agricultural Chemistry, University of Arkansas, Fayetteville)

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Peters and his associates (1) demonstrated that there is a reduced oxygen consumption of the brain of polyneuritic pigeons. Addition of minute amounts of crystalline vitamin B₁ *in vitro* to the avitaminous brain tissue restores the diminished tissue respiration. O'Brien and Peters (2) observed that the brains of vitamin B₁-deficient rats and pigeons alike produced pyruvate *in vitro* and that the presence of added vitamin B₁ removed the pyruvate. The conclusion drawn was that vitamin B₁ acts as a catalyst and is used by the tissue at some stage in the combustion of carbohydrates.

Lactic acid oxidation by normal and polyneuritic chick tissue was investigated by Sherman and Elvehjem (3) by means of oxygen uptake studies, determinations of lactic acid removal, and estimations of methylene blue reduction time. No significant disturbances in lactate oxidation were observed in brain tissue, but in the avitaminous heart a decreased rate of oxygen uptake during lactic acid removal was found. Lactate oxidation was not affected in avitaminous brain by the addition of vitamin B₁, but in the avitaminous heart similar addition produced increased oxygen uptake. Addition of pyruvate inhibited lactic acid dehydrogenase activity to a greater extent in avitaminous heart and kidney than in normal tissues.

Lipschitz, Potter, and Elvehjem (4) reported that the ability

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of the kidney, liver, and brain of chicks to utilize pyruvate as a substrate was impaired in polyneuritis, the derangement being greatest in the case of brain. The liver and kidney from fasted birds showed a decreased ability to remove added pyruvate. The brain did not show this fasting effect. Pyruvate removal by the liver and kidney of fasting birds approximated that found in polyneuritis. The oral administration of glucose to fasting birds resulted in the restoration of the ability of the liver to remove pyruvate. Similar feeding of glucose to polyneuritic birds resulted in deposition of glycogen in the liver and increased the ability to remove pyruvate, though normal values were not obtained. From these results it was concluded that inanition was not the sole cause of the deranged pyruvate metabolism, though it was certainly a complicating factor.

EXPERIMENTAL

From a review of the literature it is apparent that most of the work on tissue respiration in vitamin B₁ deficiency has been done with the pigeon and chick. The only data obtained from the rat concern the difference in oxygen uptake found in brains of normal and polyneuritic animals after addition of small amounts of crystalline vitamin B₁ *in vitro*. In the studies of oxidation-reduction efficiency during avitaminosis in our laboratory, we have, therefore, included observations of oxygen consumption in various tissues of the rat in vitamin A as well as vitamin B₁ deficiency. We used tissues of normal rats and those of avitaminotic litter mates of the same sex that were daily restricted to the same plane of nutrition as the controls. The control animals received in addition a daily supplement separately from the ration of 8 to 10 micrograms of crystalline vitamin B₁. The vitamin B₁-deficient rations had the following composition: commercial casein, 16; McCollum's Salt Mixture 185 (5), 4; Fleischmann's yeast, autoclaved for 5 hours at 15 pounds pressure, 9; beef autoclaved for 6 hours at 15 pounds pressure, 6; unfiltered butter fat, 9; cod liver oil, 2; corn-starch, 54. This ration produced loss of weight and paralysis in 2 to 3 months with great regularity. For the production, however, of convulsions, we found the following modified ration much more dependable:

casein,¹ 16; McCollum's Salt Mixture 185, 4; Fleischmann's yeast, autoclaved for 5 hours at 15 pounds pressure, 7; beef autoclaved for 6 hours at 20 pounds pressure, 8; filtered butter fat, 9; corn-starch, 56. As a source of vitamins A and D, 4 to 6 drops of cod liver oil were administered daily separately from the ration.

The vitamin A-deficient ration had the following composition: casein,² 16; Salt Mixture 185, 4; Northwestern (dehydrated bakers') yeast, 10; dextrinized starch, 66. This ration was irradiated for 30 minutes to furnish vitamin D. The control animals received 6 drops of cod liver oil or 3 drops of carotene in oil daily as a source of vitamin A.

The technique employed for the respiration studies of the various tissues was essentially that of Peters and his associates (1) with the recent modifications of Sherman and Elvehjem (3). The rats were killed by decapitation. After the blood was allowed to drain, the tissues were removed quickly and weighed in tared, small blood specimen vials containing the buffered solution of Ringer's phosphate and pyrophosphate adopted by Sherman and Elvehjem (3). The Barcroft differential respirometer was used for the oxygen uptake studies. The substrate was Merck's sodium lactate adjusted to pH 7.3 and diluted to give 3 per cent lactic acid. 0.2 ml. aliquots were added to the right- and left-hand flasks, from which an equal amount of buffer was withheld, making 0.2 per cent lactic acid in the flasks. Rolls of filter paper soaked with 8 per cent NaOH were inserted in the absorption tubes. The final volume in the Barcroft bottles was 3 ml. The brain was teased with a bone spatula, and the heart and kidney were minced with chromium-plated scissors. Oxygen was introduced from a tank into the Barcroft bottles at atmospheric pressure, since the introduction at reduced pressure produced troublesome results with the manometric fluid when the stop-cocks were opened at the end of the respiration period. The fresh tissues, weighing about 100 mg., were transferred from specimen vials to the Barcroft bottles with a loop of platinum wire. The respiration studies were carried out at 37.5°. After

¹ Washed with acidulated water for 10 days and extracted several times with 25 per cent alcohol.

² Extracted several times with hot 95 per cent alcohol.

attachment of the respiration bottles to the manometers, at least 15 minutes were allowed to elapse to insure temperature equilibrium. The taps of the apparatus were closed after the zero reading had been noted, and successive readings were taken at 30, 60, and 90 minute intervals. Each tissue was run in triplicate, and all results were averaged and expressed as oxygen uptake in microliters per mg. per hour. The variations among the triplicate determinations ranged between 2 to 10 per cent in the majority of experiments. In the few experiments, however, in which they were greater, the incidence of variation in the pathological animals was of the same degree as that observed in the controls. The apparatus was shaken uniformly at the rate of about 140 oscillations per minute. At the termination of the respiration, the tissues were dried at 103°, and all calculations were made on the dry basis.

Results

The results of this investigation are summarized in Tables I to III.

Effect of Vitamin B₁ Deficiency upon Oxygen Uptake of Cerebrum, Heart, and Kidney. *Cerebrum*—Table I shows the results of sixteen experiments on the respiration of tissues of rats in various states of polyneuritis. It is apparent that neither the extent of loss of body weight nor the degree of polyneuritis bears any direct relationship to the per cent of change in oxygen uptake of avitaminous tissue of the rat compared with that of control animals of the same litter and sex. For instance, in Experiment 3327 the pathological rat, which had no signs of polyneuritis, showed a 38 per cent reduction in oxygen uptake, while in Experiment 3598 the avitaminotic rat, which had marked paralysis and slight convulsions, showed practically no reduction in oxygen uptake. In Experiment 3531 the polyneuritic animal, which had marked paralysis and 39 per cent loss of body weight, had only a 12 per cent reduction in oxygen uptake compared with its control, while in Experiment 3433 the avitaminotic animal, which had slight paralysis and lost only 6 per cent of its body weight, showed 19 per cent reduction in oxygen uptake. The average reduction in oxygen consumption of the vitamin B₁-deficient animals compared with normal controls was 16 per cent.

TABLE I

Effect of Vitamin B₁ Deficiency upon Respiration of Rat Cerebrum in Vitro
 Oxygen uptake expressed as microliters per mg. per hour.

Experi- ment No.	Condition of animal and sex*	Loss of weight	Oxygen uptake	Change in oxygen uptake	Degree of polyneuritis
		<i>per cent</i>		<i>per cent</i>	
3433	P-L ♂	6	2.55	-19	Slight paralysis
	C ♂		3.13		
3634	P-R ♂	8	3.41	-17	Moderate paralysis; opisthotonos
	C-D ♂		4.12		
3402	P-R ♀	-15	2.54	-27	No signs of poly- neuritis
	C-D ♀		3.50		
3549	P-R ♀	19	2.37	-23	Marked paralysis
	C-D ♀		3.09		
3578	P-DL ♂	21	3.78	-7	Moderate paralysis; staggering
	C-DR ♂		4.05		
3693	P ♂	23	2.30	-21	Marked paralysis
	C-R ♂		2.94		
3554	P ♂	24	3.26	-8	Marked paralysis and convulsions
	C-L ♂		3.54		
3435	P ♀	25	3.88	+1	Marked paralysis
	C-L ♀		3.82		
3550	P-R ♂	25	3.43	-19	Marked paralysis and convulsions
	C-D ♂		4.21		
3327	P ♀	25	3.32	-38	No signs of poly- neuritis
	C-L ♀		4.13		
3598	P ♀	25	3.03	-1	Marked paralysis and slight convulsions
	C-L ♀		3.06		
3608	P-DL ♀	25	3.04	-25	Marked paralysis and slight convulsions
	C-DR ♀		4.02		
3581	P-DL ♀	31	3.01	-10	Slight paralysis
	C-DR ♀		3.31		
3580	P ♂	34	2.25	-12	Marked paralysis; opisthotonos
	C-L ♂		2.66		

TABLE I—*Concluded*

Experi- ment No.	Condition of animal and sex*	Loss of weight	Oxygen uptake	Change in oxygen uptake	Degree of polyneuritis
		<i>per cent</i>		<i>per cent</i>	
3531	P-L ♀	39	2.23	—12	Marked paralysis
	C ♀		2.49		
3513	P-Dl ♀	41	2.81	—21	Slight paralysis
	C-DR ♀		3.58		
Average, 16 groups.....				—16	

* P = pathological; C = control; L = left slit; R = right slit; DL double left slit; D = double slit; DR = double right slit.

Heart and Kidney—Fourteen groups of animals were used for the heart and nineteen groups for the kidney respiration studies. The average reduction in the oxygen uptake of the avitaminous tissues was 17 and 19 per cent, respectively, compared with normal controls. Here again there was no correlation in the polyneuritic state, the extent of loss of weight, and the degree of oxygen uptake. Detailed data are presented in Tables II and III.

Oxygen Uptake of Kidney and Heart in Deficiency of Vitamin B Complex—In addition to the studies made during vitamin B₁ deficiency, twenty-one respiration experiments were carried out with kidney and nine with heart from animals that were on diets deficient in all the components of the vitamin B complex. In order to conserve space, tabular data are omitted. The avitaminous kidneys showed an average reduction of 15 per cent in oxygen uptake and the avitaminous heart 18 per cent, compared with tissues from normal control animals. Extent of loss of weight was not a factor influencing reduction of oxygen uptake.

Influence of Vitamin A Deficiency upon Oxygen Uptake of Cerebrum and Heart—Respiration experiments were also carried out during vitamin A deficiency, thirteen groups of animals having been used for oxygen uptake of cerebrum tissue and seven groups for heart tissue. To conserve space, detailed tabular data are omitted. We have observed cases of pathological animals with advanced ophthalmia that showed normal respiration of cerebrum compared with tissue of control animals. On the other hand,

TABLE II

Effect of Vitamin B₁ Deficiency upon Respiration of Rat Heart in Vitro
 Oxygen uptake expressed as microliters per mg. per hour.

Experiment No.	Condition of animal and sex*	Loss of weight	Oxygen uptake	Change in oxygen uptake	Degree of polyneuritis
		<i>per cent</i>		<i>per cent</i>	
3407	P-R ♂	9	1.51	-8	Marked paralysis and slight convulsions
	C-D ♂		1.70		
3465	P-R ♀	16	1.20	-11	Moderate paralysis
	C-D ♀		1.35		
3420	P-DL ♂	18	1.54	+5	Marked paralysis; staggering
	C-DR ♂		1.47		
3412	P ♀	22	1.69	-15	Marked paralysis and slight convulsions
	C-L ♀		2.00		
3659	P-DL ♂	23	2.31	-16	Marked paralysis and convulsions
	C-D ♂		1.98		
3515	P-R ♂	23	0.82	-40	Marked paralysis and staggering
	C-D ♂		1.37		
3439	P ♂	24	1.31	-23	Marked paralysis and convulsions
	C-L ♂		1.68		
3656	P ♂	26	2.59	-13	Marked paralysis
	C-L ♂		2.97		
3548	P ♂	27	2.46	+2	Slight convulsions
	C-L ♂		2.41		
3653	P-R ♀	27	2.99	-10	No signs of polyneuritis
	C-D ♀		3.38		
3509	P-R ♂	27	0.66	-56	Slight paralysis
	C-D ♂		1.50		
3508	P-D ♀	29	2.24	-10	Slight paralysis
	C-R ♀		2.50		
3514	P ♀	35	2.01	-16	Staggering gait
	C-L ♀		2.41		
Average, 13 groups.....				-17	

* See explanation of symbols below Table I.

TABLE III

Effect of Vitamin B₁ Deficiency upon Respiration of Rat Kidney in Vitro
 Oxygen uptake expressed as microliters per mg. per hour.

Experiment No.	Condition of animal and sex*	Loss of weight	Oxygen uptake	Change in oxygen uptake	Degree of polyneuritis
		<i>per cent</i>		<i>per cent</i>	
3388	P-R ♂	16	2.39	-10	No signs of polyneuritis
	C-D ♂		2.67		
3577	P-D ♀	18	2.99	-22	Marked paralysis
	C-R ♀		3.77		
3403	P ♀	19	5.80	-21	Marked paralysis
	C-L ♀		7.26		
3383	P-R ♂	24	3.00	-16	Slight paralysis
	C-D ♂		3.56		
3411	P ♂	24	2.98	-8	No signs of polyneuritis
	C-L ♂		3.23		
3599	P-D ♂	24	6.32	-14	Marked paralysis; convulsions
	C-R ♂		7.32		
3366	P-DL ♀	26	2.85	-5	Slight paralysis
	C-DR ♀		3.01		
3652	P-L ♀	26	4.00	-24	Slight paralysis
	C ♀		5.28		
3606	P-DL ♂	27	3.85	-31	Marked paralysis
	C-DR ♂		5.57		
3636	P-DR ♂	28	4.38	-41	Marked paralysis
	C-DL ♂		7.38		
3345	P-R ♂	29	2.34	-22	Marked paralysis
	C-D ♂		3.02		
3389	P-D ♂	30	2.43	-24	No signs of polyneuritis
	C-DR ♂		3.19		
3306	P-D ♂	30	2.56	-28	No signs of polyneuritis
	C-DR ♂		3.56		
3307	P ♀	33	3.33	-17	No signs of polyneuritis
	C-DL ♀		4.02		
3325	P-L ♀	34	2.81	-4	No signs of polyneuritis
	C-D ♀		2.92		

TABLE III—*Concluded*

Experiment No.	Condition of animal and sex*	Loss of weight	Oxygen uptake	Change in oxygen uptake	Degree of polyneuritis
		<i>per cent</i>		<i>per cent</i>	
3269	P-R ♀	34	4.54	-22	Marked paralysis
	C-D ♀		5.85		
3360	P ♂	36	2.67	-19	Marked paralysis
	C-L ♂		3.32		
3380	P-DL ♀	36	2.59	-20	Marked paralysis
	C-DR ♀		3.22		
3361	P-R ♂	36	3.04	-17	Slight paralysis
	C-D ♂		3.66		
Average, 19 groups.....				-19	

* See explanation of symbols below Table I.

animals with only slight ophthalmia in one eye gave a reduction of 32 per cent of oxygen uptake. No correlation was found between the intensity of vitamin A deficiency, as is evidenced by the severity of ophthalmia, labored respiration, and loss of weight, and reduction of oxygen uptake of avitaminous cerebrum compared with the same tissue from controls. In the case of the heart, however, too few animals were used to warrant any correlation between the avitaminotic state and efficiency of respiration. It is interesting to note that the average reduction in oxygen consumption of cerebrum of thirteen groups of vitamin A-deficient animals (14 per cent) was very near the reduction observed in sixteen groups of cerebrum of animals deficient in vitamin B₁ (16 per cent). Also, the average reduction in oxygen uptake of hearts of seven groups of vitamin A-deficient animals (19 per cent) was approximately the same as that observed in fourteen groups of vitamin B₁-deficient animals (17 per cent).

DISCUSSION

The oxygen uptake studies reported in this communication constitute a continuation of our investigations on enzymic efficiency in avitaminosis (6). Since the oxygen uptake of tissues furnishes information of the sum total of activity of the various individual oxidase systems, provided a plentiful supply of substrate is sup-

plied (3), it was anticipated that various pathological states in vitamin B₁ and vitamin A deficiencies would result in quantitative correlations between the amount of oxygen consumed by tissues and the degree of intensity of the specific symptoms of avitaminosis. Such a correlation, however, was not found to exist in our studies.

In the recent review of the physiology of vitamin B₁, Cowgill (7) refers to observations made in Dr. A. B. Hastings' laboratory at Harvard to the effect that, in the presence of vitamin B₁ deficiency, the tissue of the auricle, in contrast to that of the ventricle, shows a marked reduction in oxygen uptake when compared with the normal. Such results, Dr. Cowgill believes, constitute proof that these two parts of the heart respond differently to a deficiency of vitamin B₁ and that the auricle is more sensitive in this respect than the ventricle. One would then expect that the administration of vitamin B₁ would restore the lost tone of the heart. As a matter of fact, the beneficial effect of vitamin B₁ in cardiac diseases has recently been reported by Weiss and Wilkins (8) and Jones and Sure (9).

That there is a disturbance in carbohydrate metabolism in vitamin B₁ deficiency has been conclusively demonstrated and that the derangement takes place in the oxidation of pyruvic acid is also clear. Since the recent demonstration by Lohmann and Schuster (10) that cocarboxylase is a diphosphoric ester of vitamin B₁, the interpretation suggests itself that the disappearance of pyruvate following minute vitamin B₁ additions is associated with a decarboxylation process resulting in acetaldehyde and carbon dioxide as intermediate steps in carbohydrate metabolism, most probably accompanied by an additional reaction involving the production of acetic acid and carbon dioxide.

That reduced oxygen uptake is not characteristic and specific for vitamin B₁ deficiency is evident from the report of Adams (11) who found that the oxygen consumption of the skin of vitamin G-deficient rats declines to a much lower level than in normal rats of the same age, and from the report of Presnell (12) who observed that the oxygen uptake of skins of vitamin D-deficient rats was only 60 to 70 per cent of that of normal controls.

The mechanism involved in the reduction of oxygen uptake of

brain and heart in vitamin A deficiency is open for investigation. The most puzzling question in our minds is the lack of association of oxygen uptake of tissues and the avitaminotic state of the animal. Undoubtedly there must be many factors influencing oxygen consumption of tissues of vitamin-deficient animals that are not all clear and which require further study.

SUMMARY

Measurements were made with the Barcroft apparatus of the oxygen uptake of several tissues of the albino rat. The experiments were carried out by the paired feeding method, the daily food intake of the control animals having been restricted to the same plane of nutrition as that consumed by the pathological litter mates during the previous 24 hours. The results, expressed as per cent reduction in oxygen uptake, can be summarized as follows:

Deficiency of the vitamin B complex: kidney, twenty-one groups, -15 per cent; heart, nine groups, -18 per cent. Vitamin B₁ deficiency associated with polyneuritis: cerebrum, sixteen groups, -16 per cent; heart, fourteen groups, -17 per cent; kidney, nineteen groups, -19 per cent. Vitamin A deficiency: cerebrum, thirteen groups, -14 per cent; heart, seven groups, -19 per cent. Neither the extent of loss of weight, the severity of polyneuritis, nor the severity of ophthalmia had any influence on the quantitative reduction of oxygen uptake of either the kidney, heart, or cerebrum of the albino rat on diets deficient in vitamin B complex, vitamin B₁, or vitamin A, respectively.

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THE MALE SEX HORMONES OF HUMAN URINE AND BLOOD

BY D. ROY McCULLAGH AND W. O. OSBORN

(From the Department of Biochemical Research, Cleveland Clinic, Cleveland)

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Adler (1) in 1934 demonstrated that a physiologically inactive extract of human male urine can be obtained with butyl alcohol and that, by subsequent heating with strong acid, the inactive extract can be converted into a potent material which promotes the growth of the combs of capons. Peterson, Gallagher, and Koch (2) in 1937 confirmed the acid hydrolysis of the inactive form and have determined the optimum time of hydrolysis by boiling urine acidified with one-tenth by volume of commercial hydrochloric acid. It is well known that the active forms of the comb growth-promoting substances are water-insoluble and lipid-soluble. Dingemanse, Borchardt, and Laqueur (3) in 1937 demonstrated that the inactive form is water-soluble and lipid-insoluble. They have also discovered the important facts that the inactive form is not made lipid-soluble by even prolonged boiling with strong aqueous or alcoholic potassium hydroxide and that the latent activity is not destroyed by such treatment, for, if followed by acid hydrolysis, the active form is completely recoverable, as shown in tests made by injection into capons. Dingemanse and Laqueur (4) have studied methods of avoiding destruction of androgens while activating the inactive form with hydrochloric acid. Recently, Peterson, Hoskins, Coffman, and Koch (5) have found that normal butyl alcohol extracts all the androgenic material from male urine and that this material is practically all in an inactive form. At the same time McCullagh, Osborn, and Osgard (6) made a similar preliminary report of the experiments discussed in this paper.

Experiments designed to throw further light on the properties of the free and inactive androgens in urine have been carried out

in this laboratory by use of the rat assay method of Korenchevsky (7), the capon injection assay method of Gallagher and Koch (8), and also the capon comb inunction assay method of Fussgänger (9). McCullagh and McLin (10) showed that quantitative extraction of free androgens from urine can readily be made by the use of dibutyl ether. The method of separation of free and inactive androgens was based upon that work combined with the fact established in this paper that there is no appreciable extraction of inactive urinary androgens by the use of this solvent.

McCullagh, McCullagh, and Hicken (11) were the first to point out the existence of androgens in human blood. The hormone content of blood was obtained by ether extraction of the solid mass resulting from the desiccation of the blood with anhydrous sodium sulfate. The possible existence of an inactive androgen in blood was not suspected at that time but definite evidence is presented in this paper to prove the existence of such a compound, and to show it to be insoluble in dibutyl ether.

EXPERIMENTAL

In preliminary experiments, urine from unemployed men who were being fed and housed by the city of Cleveland was examined for androgens and for substances which become androgenic following boiling with acid. In these experiments extraction with dibutyl ether showed that this urine contained little if any active androgenic material. The urine was extraordinarily low in total androgenic material, containing only 7 international units per liter after boiling for 15 minutes with 5 per cent sulfuric acid. The assays were made by the methods of Korenchevsky (7) and of Gallagher and Koch (8). These methods necessitated the use of rather large samples of urine and hence an extended period of extraction. There was indication that some activation occurred during this time.

Therefore, freshly excreted urine from four normal men working in this institution was studied. In order to make the extractions more rapid, small quantities of urine were used and the extracts were assayed according to the inunction method on the combs of capons. In each case, 150 cc. of freshly voided urine were acidified by the addition of 7.5 cc. of concentrated sulfuric acid and extracted twice by shaking vigorously for 10 minutes with an

equal volume of cold dibutyl ether. The two dibutyl ether extracts were combined, washed with water, and evaporated to dryness by steam distillation in a partial vacuum. The oily residue was dissolved in 6 cc. of sesame oil. 60 cc. of the extracted urine were then boiled for 15 minutes, cooled under running water, and reextracted in a similar fashion. Five birds were used to assay each preparation; 0.1 cc. of oil was applied to each side of the comb daily for 5 days. The combs were measured at the beginning of the experiment and again on the 7th day. Table I gives the results of this study. In another publication (12) the dose-response curve of the capon comb following the administration of androsterone is given. The experiments show that contrary to the opinions expressed by earlier workers the specimens

TABLE I

Androgens in Normal Male Human Urine, Expressed As International Units

Specimen No.	Androgens per liter before boiling with acid	Androgens per liter after boiling with acid
1	0.5	38
2	0	49
3	0	45
4	0	46

of fresh urine examined contained no measurable androgenic material. The same urine boiled for 15 minutes with 5 per cent sulfuric acid by volume contained an average of 44 international units per liter of dibutyl ether-soluble androgen. It seems probable that the more drastic methods of extraction employed by other workers caused partial activation of the hormone during the process of extraction.

Further similar tests show that the inactive hormone is insoluble in benzene, chloroform, and dibutyl ether. It is quite soluble in butanol, slightly soluble in ethyl acetate, and can to a large extent be adsorbed on charcoal.

Androgens in Blood

Various methods have been used in this laboratory for the extraction of androgenic material from blood. The most effective is the following simple procedure.

30 cc. of oxalated blood are laked with 270 cc. of water and 15 cc. of concentrated sulfuric acid. The mixture is boiled for 15 minutes and thoroughly extracted twice with 150 cc. quantities of dibutyl ether. Emulsions which may form are broken down by the addition of sodium taurocholate. The dibutyl ether is washed twice with 50 cc. of 10 per cent sodium hydroxide and twice with 50 cc. of water and evaporated to dryness by steam distillation in a partial vacuum. The residue is removed from the flask to a small beaker with ethyl ether; 5 cc. of sesame oil are added and the ether removed on a water bath. The oil is applied evenly over the combs of five birds over a period of 5 days; *i.e.*, each bird receives 0.1 cc. of oil on each side of the comb each day. The increase in comb size is measured 24 hours after the final inunction. The results can be expressed in international units after interpretation according to the dose-response curve.

Twenty assays carried out on a total of 100 capons have already been made. It has been found that the extracts of 50 cc. of fresh human male blood which has not been boiled caused no comb growth in five birds. Mixed samples standing at room temperature for some hours contain traces of free androgenic material. The greatest yield was obtained after boiling for 15 minutes, although boiling for shorter periods causes some activation. Continued boiling causes a rapid decrease in androgenic content. Boiling for 30 minutes decreases the yield about 50 per cent. Some androgenic activity remains after boiling for 5 hours. With the method suggested above, about 4 international units are obtained from 100 cc. of blood from normal young men.

SUMMARY

1. All the androgenic material in freshly voided male human urine is in an inactive form; androgens are present after boiling with acid.

2. The inactive androgenic substance is soluble in butanol, less soluble in ethyl acetate, and insoluble in benzene, chloroform, and dibutyl ether. It is adsorbed by charcoal.

3. A method is described for the extraction and assay of androgens in blood.

4. All the androgenic material in normal male human blood is inactive and can be activated by boiling with acid.

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METABOLIC INVESTIGATIONS ON A CASE OF PHENYLPYRUVIC OLIGOPHRENIA*

By GEORGE A. JERVIS

*(From the Research Department, Letchworth Village, New York State
Department of Mental Hygiene, Thiells)*

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An apparently new type of metabolic abnormality in man has been described recently by Fölling (1). This author reports having found phenylpyruvic acid in the urine of ten mentally defective patients. Shortly thereafter, similar cases were discovered by Penrose (2) in England, and by the writer (3) in the United States. The term phenylpyruvic oligophrenia has been proposed for this condition. The present paper records the effects of feeding various compounds to a patient afflicted with this peculiarity.

The subject, B. J., was a well developed and otherwise healthy male imbecile, aged 28 years. During the experiments he received two basal diets. The first (Diet A) was nitrogen-free. It consisted of 300 gm. of corn-starch and 150 gm. of sugar a day. The daily urinary excretion of nitrogen was thus reduced to 2 to 3 gm., and the phenylpyruvic acid output to 250 to 350 mg. The second diet (Diet B) consisted of Cream of Wheat 100 gm., bread 130 gm., and milk 400 gm. for breakfast; and two eggs, potatoes 225 to 230 gm., bread 150 gm., milk 400 gm., and two oranges for both lunch and supper. The daily excretion of nitrogen and phenylpyruvic acid was constant within a range which was considered sufficiently satisfactory, the value of nitrogen ranging from 14 to 15 gm., and the value of phenylpyruvic acid from 1.8 to 2.1 gm.

Methods

All determinations were performed on 24 hour specimens of urine. Total nitrogen was determined by the usual Kjeldahl

* Aided by a grant from Child Neurology Research (Friedsam Foundation).

method, urea according to the urease method of Van Slyke and Cullen, and ammonia with Folin's aeration procedure. The pH, amino acid nitrogen, and creatinine were determined colorimetrically. The figures for urea, ammonia, preformed creatinine, and pH showed nothing of significance, and have been omitted from the tables.

For the quantitative determination of phenylpyruvic acid, the colorimetric method of Penrose and Quastel (4) was used. In addition, a gravimetric technique was worked out, based on the capacity of 2,4-dinitrophenylhydrazine to form insoluble hydrazones with keto acids (Neuberg and Kobel (5)). To 100 cc. of filtered urine, 300 mg. of 2,4-dinitrophenylhydrazine dissolved in 5 cc. of hot 2 N HCl were added. A precipitate of dinitrophenylhydrazone formed immediately. The mixture, after standing for several hours at room temperature, was extracted with ether. After evaporation of the ether, the hydrazone was dissolved in a small amount of saturated sodium carbonate and reprecipitated by acidification. The precipitate was removed by filtration through a Jena crucible, washed with 200 cc. of warm 2 N HCl in order to remove residua of hydrazine, and then with 200 cc. of water. It was dried overnight in an oven at 80° and weighed.

A considerably easier method, based upon the capacity of phenylpyruvic acid to combine, molecule for molecule, with sodium bisulfite (Hemmerlé (6)), was also used in all determinations, but was not so accurate as the two procedures mentioned above. The oxidation method described in a previous paper (3) yielded constantly higher figures, since hippuric and other chloroform-soluble acids cannot be completely eliminated.

Administration of Phenylalanine

The racemic and the two optically active forms of phenylalanine (Hoffmann-La Roche) were repeatedly fed, the patient being kept on both Diets A and B. After each feeding, 100 cc. of urine, from which phenylpyruvic acid had been removed by precipitation as the hydrazone, were extracted with butyl alcohol. The alcohol was evaporated, and the dry residuum was tested for phenylalanine according to the colorimetric method of Kappeler-Adler (7). In no case was phenylalanine detected.

As seen in Table I, a rise in the urinary output of phenyl-

pyruvic acid constantly followed the ingestion of phenylalanine. This observation, which was originally reported by Fölling (1), appears of basic importance. There is convincing evidence, derived from feeding experiments (Kotake (8), Chandler and Lewis (9)) and from oxidation studies *in vitro* with tissue slices (Krebs (10), Neber (11), Lieben and Kretschmayer (12)) in support of the assumption that phenylpyruvic acid is an inter-

TABLE I
Feeding of Phenylalanine

Form of compound fed (5 gm.)	Date	Total N	Amino acid N	Total creatinine	Phenylpyruvic acid		Ratio, phenylalanine to N
					Colorimetric	Hydrazine	
		gm.	gm.	gm.	gm.	gm.	
<i>d</i>	May 16-17	15.5	0.24	1.37	1.96	2.07	12.9
	" 18	16.1	0.31	1.31	3.28	2.96	19.3
	" 19	14.9	0.18	1.39	2.25	2.14	14.7
	" 20	14.3	0.16	1.27	1.77	1.78	12.4
<i>l</i>	June 16-17	13.9	0.16	1.44	1.70	1.71	12.3
	" 18	14.0	0.29	1.42	2.40	2.34	16.9
	" 19	14.6	0.16	1.49	2.41	2.37	16.3
	" 20	15.5	0.16	1.39	2.04	2.00	13.0
	" 24	13.9	0.17	1.39	1.79	1.97	13.5
<i>dl</i>	" 25	16.4	0.29	1.40	3.04	2.84	17.8
	" 26	15.0	0.19	1.43	2.16		14.4
<i>d</i>	Dec. 13	4.1	0.13	1.62	0.63	0.88	18.4
	" 14	3.6	0.18	1.17	1.30	1.37	37.1
	" 15-16	3.6	0.10	1.32	0.52	0.75	17.6
<i>l</i>	" 17	3.9	0.11	1.50	0.91	1.28	28.1
	" 18	3.3	0.13	1.50	0.78	0.81	24.1
<i>dl</i>	" 19	3.7	0.12	1.55	0.50	0.76	17.0
	" 20	3.7	0.16	1.28	1.01	1.05	27.8
	" 21	3.8	0.19	1.38	0.63	0.74	18.0

mediary step in the metabolism of phenylalanine. It is, therefore, reasonable to assume that the phenylpyruvic acid present in the urine of the patient was derived from the incomplete oxidation of the amino acid.

Following the ingestion of other pure amino acids, including glycine 20 gm., alanine 10 gm., valine 10 gm., leucine 10 gm., cystine 10 gm., and tryptophane 4 gm., no increased excretion of the ketonic acid was observed (Table II).

That phenylalanine is the source of the phenylpyruvic acid appears also from the following experiments. Proteins containing various amounts of phenylalanine were fed, the content of phenylalanine of each having been determined by the Kappeler-Adler method (7): gelatin, casein, and edestin 100 gm. each, representing 0.4, 4.4, and 3.8 gm. of phenylalanine respectively. As shown in Table III, the higher the phenylalanine content of the protein, the greater the increase in output of phenylpyruvic acid.

TABLE II
Feeding of Amino Acids

Compound fed	Date	Total N	Amino acid N	Total creatinine	Phenylpyruvic acid		Ratio, phenylalanine to N
					Colorimetric	Hydrazine	
		gm.	gm.	gm.	gm.	gm.	
Glycyl- <i>dl</i> -phenylalanine, 3.5 gm.	Mar. 6	14.3		1.35	1.74	1.70	12.0
	" 7	15.1		1.34	2.33	2.42	15.7
	" 8	16.0		1.40	2.00	2.00	12.5
Phenyl serine, 5 gm. Ureido, 5 gm.	Jan. 10	3.5	0.11	1.40	0.38	0.47	12.1
	" 11	4.6	0.12	1.40	0.46	0.51	10.5
	" 12	4.0	0.14	1.33	0.35	0.40	9.4
Glycine, 20 gm.	Sept. 28	16.5	0.15	1.39	1.98	2.03	12.2
	" 29	18.6	0.19	1.39	2.05	2.02	10.9
	Oct. 5	15.7	0.17	1.34	1.90	2.00	12.4
Alanine, 10 gm.	" 6	14.3	0.26	1.30	1.90	1.92	13.4
	" 7	15.8	0.28	1.52	1.88	2.12	12.7
	" 8	15.5	0.36	1.41	1.98	1.88	12.5
Valine, 10 gm.	" 9	16.0	0.48	1.50	2.14	1.90	12.6
Leucine, 10 gm.	" 10	16.3	0.22	1.53	1.69	2.13	11.7
Cystine, 10 "	" 21	3.0	0.07	1.34	0.36	0.37	12.2
Tryptophane, 4 gm.	" 22	2.9	0.06	1.41	0.40	0.41	14.0

When the deamination of phenylalanine is blocked, no increase in the urinary output of phenylpyruvic acid results, as is shown in feeding experiments with the ureido derivative of phenylalanine (Table II). Negative results were also obtained following the ingestion of β -hydroxyphenylalanine in which the presence of the β -hydroxyl group prevents α oxidation (Dakin (13)). On the other hand, the ingestion of glycyl-*dl*-phenylalanine is followed by an increased output of phenylpyruvic acid.

It will be noted that the output of phenylpyruvic acid was not

equivalent to the total amount of phenylalanine fed. It appears, therefore, that part of the amino acid was completely oxidized, since none was recovered as such in the urine. This finding may be explained either by an alternative path in the oxidation of phenylalanine through tyrosine (Embden and Baldes (14), Kotake (8), Medes (15), Edson (16)), or by the assumption that the metabolic error in our subject was not complete.

The study of the behavior of the two optically active forms of phenylalanine appears of some interest. It will be noted (Table I) that on both diets the excretion of phenylpyruvic acid was greater after feeding the *d* form than after feeding the *l* form. These results appear to indicate that the naturally occurring *l*-phenylalanine is utilized more readily than its optical isomer, as has been

TABLE III
Feeding of Various Proteins

Compound fed (100 gm.)	Date	Total N	Amino acid N	Total creatinine	Phenylpyruvic acid		Ratio, phenylalanine to N
					Colorimetric	Hydrazine	
		gm.	gm.	gm.	gm.	gm.	
Gelatin	Mar. 9	14.9	0.17	1.37	1.95	1.94	13.1
	" 10	21.5	0.16	1.40	2.06	2.09	9.2
	" 17	15.9	0.18	1.42	2.05	1.84	12.2
Casein	" 18	24.6	0.25	1.52	2.67	2.75	11.0
	" 21	14.8	0.14	1.35	1.81	1.68	11.8
Edestin	" 22	21.5	0.24	1.43	2.35	2.27	10.8

shown to be the case in the normal human organism by Kotake *et al.* (17), and in animals by Chandler and Lewis (9).

The formyl and acetyl derivatives of both forms of phenylalanine, prepared according to the directions of du Vigneaud and Meyer (18), were also fed to the patient. As is shown in Table IV, the formyl and acetyl derivatives of *d*-phenylalanine are not oxidized to the stage of phenylpyruvic acid, whereas the derivatives of the natural enantiomorph undergo oxidation in the organism, since they yield a small but constant increase in the output of phenylpyruvic acid. These results appear in agreement with evidence obtained in growth experiments (du Vigneaud *et al.* (19), Jackson and Block (20)), indicating that the hydrolytic

reaction which occurs before the utilization of an amino acid is extremely specific with respect to spatial configuration.

Effects of Phenylpyruvic Acid and Certain Related Compounds

Phenylpyruvic acid, prepared according to the directions of Hemmerlé (6), was fed repeatedly. A rapid rise in the urinary output of the acid, and a marked increase in the ratio of phenylpyruvic acid to nitrogen, constantly followed (Table V). It seems confirmed, therefore, that this acid was broken down with difficulty in the organism of the patient. An increase in the out-

TABLE IV
Feeding of Formyl- and Acetylphenylalanine

Compound fed (5 gm.)	Date	Total N	Amino acid N	Total creatinine	Phenylpyruvic acid		Ratio, phenylalanine to N
					Colorimetric	Hydrazine	
		gm.	gm.	gm.	gm.	gm.	
Formyl- <i>d</i> -phenylalanine	Aug. 2	2.7	0.05	1.27	0.33	0.31	11.9
	Mar. 3	2.8	0.06	1.24	0.29	0.32	10.9
	" 4	2.7	0.06	1.32	0.32	0.33	12.0
Formyl- <i>l</i> -phenylalanine	Feb. 5	2.6	0.06	1.05	0.48		18.5
	" 6	2.9	0.06	1.09	0.41	0.44	14.7
	" 7	2.4	0.05	1.08	0.33	0.29	12.9
Acetyl- <i>l</i> -phenylalanine	" 8	2.4	0.06	1.10	0.47	0.49	20.0
	" 9	2.4	0.06	0.94	0.45	0.46	18.9
	" 10	2.5	0.06	1.15	0.35	0.43	15.6
Acetyl- <i>d</i> -phenylalanine	" 11	2.6	0.07	1.06	0.31	0.36	12.9

put of phenylpyruvic acid occurred also following the ingestion of phenyllactic acid (5 gm.), whereas no increase was obtained with the phenyl derivatives of other fatty acids containing 3 carbon atoms in the aliphatic chain (Table V).

Influence of Tyrosine and Tyrosine Derivatives

Tyrosine (Hoffmann-La Roche) in doses of 10 and 25 gm. was without influence upon the urinary excretion of phenylpyruvic acid. Nor were increases observed following the administration of diiodotyrosine 4 gm., glycylytyrosine 4 gm., dihydroxyphenylalanine 3 gm., homogentisic acid 3 gm., and *p*-hydroxyphenylpyruvic acid 3 gm. (Table VI). In each of these experiments the urine

TABLE V
Feeding of Phenylpyruvic Acid and Other Compounds

Acid fed (5 gm.)	Date	Total N	Amino acid N	Total creatinine	Phenylpyruvic acid		Ratio, phenylalanine to N
					Colorimetric	Hydrazine	
		gm.	gm.	gm.	gm.	gm.	
Phenylpyruvic	Jan. 12	4.0	0.19	1.33	0.35	0.42	9.6
	" 13	3.8	0.16	1.23	1.62	1.40	39.8
	" 14	3.2	0.16	1.15	0.54	0.61	18.0
	" 15	3.2	0.20	1.17	0.45		14.0
Phenylpyruvic	Sept. 13	15.6	0.14	1.37	1.89	1.86	12.0
	" 14	16.5	0.15	1.36	3.53	3.92	22.6
	" 15	17.1	0.15	1.43	2.20	1.90	11.9
	" 16	17.1	0.15	1.58	2.00	1.95	11.5
Phenyllactic	Jan. 19	3.5	0.10	1.29	0.36	0.34	10.0
	" 20	3.6	0.10	1.17	0.98	0.79	24.6
	" 21	2.9	0.07	1.23	0.52	0.32	14.5
Phenylglyceric	" 8	3.6	0.10	1.33	0.42	0.62	14.4
	" 9	3.7		1.48	0.46	0.63	14.7
	" 10	3.5	0.11	1.40	0.36		10.3
Phenylpropionic	Apr. 30	16.5	0.18	1.55	1.82	2.13	11.9
	May 1	16.6	0.17	1.44	2.00	2.01	12.1
	" 2	15.0	0.14	1.43	1.93	1.93	12.9
Cinnamic	June 28	15.9	0.22	1.36	1.75		11.0
	" 29	16.1	0.21	1.28	1.73	1.80	10.9
	" 30	15.6	0.14	1.57	1.94	2.09	12.9
Phenylacetic	Apr. 19	15.0	0.18	1.50	2.13		14.2
	" 20	17.2	0.20	1.58	1.84	1.83	10.6
	" 21	16.5	0.23	1.48	2.12	2.00	12.5

TABLE VI
Feeding of Tyrosine and Derivatives

Compound fed	Date	Total N	Amino acid N	Total creatinine	Phenylpyruvic acid	
					Colorimetric	Hydrazine
		gm.	gm.	gm.	gm.	gm.
Tyrosine, 25 gm.	July 27	16.0		1.60	1.85	2.03
	" 28	17.0		1.58	1.90	1.95
	Oct. 18	4.1	0.09	1.10	0.36	0.35
Homogentisic acid, 3 gm. Dihydroxyphenylalanine, 3 gm.	" 19	4.2	0.10	1.40	0.42	0.44
	" 20	3.8	0.11	1.42	0.42	0.40
Diiodotyrosine, 4 gm. Glycyltyrosine, 4 "	" 22	2.8	0.07	1.41	0.40	0.40
	" 23	2.5	0.06	1.38	0.35	0.42
Hydroxyphenylpyruvic acid, 3 gm.	Mar. 3	13.4		1.26	1.76	1.74
	" 4	15.4		1.69	2.17	2.10
	" 5	14.3		1.36	1.82	

was tested for the presence of tyrosine and its catabolites by the Millon method, as described by Fürth and Scholl (21). No significant results were obtained in either the ether-soluble or the ether-insoluble fractions. The findings indicate, as would be expected theoretically, that tyrosine does not yield phenylpyruvic acid. Moreover, since no catabolites of tyrosine were recovered, it seems likely that the patient experienced no difficulty in utilizing this amino acid.

SUMMARY

1. Feeding experiments to a patient with phenylpyruvic oligophrenia are reported. The following pure amino acids were fed: phenylalanine, tyrosine, tryptophane, phenylserine, dihydroxyphenylalanine, alanine, leucine, cystine, valine, and glycine. Of these only phenylalanine increases the urinary output of phenylpyruvic acid.

2. The dextro form of phenylalanine induces a greater increase in phenylpyruvic acid than the levo form. Formyl- and acetyl-*D*-phenylalanine do not yield phenylpyruvic acid, whereas the corresponding derivatives of *L*-phenylalanine increase the excretion of phenylpyruvic acid.

3. The ingestion of phenylpyruvic and phenyllactic acids leads to increased excretion of phenylpyruvic acid. Phenylpropionic, phenylglyceric, cinnamic, *p*-hydroxyphenylpyruvic, and homogentisic acids fail to augment the output of phenylpyruvic acid.

4. The significance of these findings is briefly discussed. It is concluded that the disease is characterized biochemically by an inhibition in the metabolism of phenylalanine at the stage of phenylpyruvic acid, the subject being unable to oxidize this keto acid at a normal rate.

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CHANGES OF NITROGEN CONTENT BROUGHT ABOUT BY DENATURATION OF PROTEINS*

BY BYRON M. HENDRIX AND JOE DENNIS

(From the Laboratory of Biological Chemistry, School of Medicine, University
of Texas, Galveston)

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There are a few papers in the literature which indicate a slight difference in the nitrogen content of a native protein and its corresponding denatured derivative. Osborne (1) found the nitrogen content of edestin to be 18.69 per cent, while edestan, a denatured edestin, contained 18.50 per cent nitrogen. Florence, Enselme, and Pozzi (2) found similar preparations to differ in this respect. Their edestin contained 18.71 per cent and the denatured material 17.92 per cent nitrogen. If these differences are real, they could indicate a splitting of nitrogen-rich materials from the protein molecule by the denaturation process or an addition of water to the molecule in the amount necessary to produce the observed lowering.

In order to see whether there is a statistically significant alteration of the nitrogen content of proteins accompanying their denaturation we have prepared and analyzed, for nitrogen, samples of egg albumin and edestin along with their corresponding denatured derivatives. In order that the results could be treated statistically we have made a large number of determinations on each preparation, the number presented in this paper varying from 105 in the case of one of the crystalline albumins to 41 in the case of the other crystalline albumin. We have felt that this number of determina-

* The data in this paper have been taken from a thesis presented by Mr. Dennis to the Graduate School of the University of Texas in partial fulfillment of the requirements for the degree of Master of Arts. A preliminary report has been made at the meeting of the American Society of Biological Chemists at Baltimore, March, 1938 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. liii (1938)).

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tions is sufficient for statistical purposes. Additional preparations have been made and analyzed but for purposes of brevity are not included in this report. To check the possibility that nitrogen-rich materials (ammonia, etc.) were split from the molecule by the treatment employed in preparing the denatured samples we have analyzed in every case the filtrates from these preparations.

Methods

The egg albumin used in these experiments was prepared and twice recrystallized by a slight modification of the method of Kekwick and Cannan (3). The samples thus prepared consist of a mixture of the crystalline protein and sodium sulfate. This mixture was analyzed for moisture, ash, and nitrogen, and the nitrogen figures given are calculated to the moisture- and ash-free basis.

The alkali-denatured albumins were prepared by dissolving the crystalline material in water (usually 300 gm. in 4 liters of water) and adding 1.0 N sodium hydroxide until the total alkalinity was 0.1 N. After this material had stood for stated periods of time (30 minutes to 6 hours), the solutions were adjusted to the isoelectric point with 1.0 N hydrochloric acid and the insoluble denatured protein filtered off, washed thoroughly with water, and then dried either by allowing the water to evaporate, or with alcohol and ether. The washings and filtrates were combined and analyzed as outlined below. The acid-denatured albumin was obtained by adjusting solutions of crystalline albumin to about pH 1 with hydrochloric acid and permitting them to stand overnight. The protein was precipitated by adjusting to the isoelectric point with 1.0 N sodium hydroxide and filtered, washed, and dried as described above. A denatured albumin was also produced by shaking solutions of the crystalline material until a small aliquot of the solution gave only a faint turbidity with trichloroacetic acid. These samples were washed and dried in the same manner as the other denatured products.

The edestin used was prepared and twice recrystallized by the method of Osborne (4). The crystalline material was dried in a vacuum desiccator over KOH at 2°. A sample of edestan was prepared by allowing 185 gm. of this product to stand for 2 weeks

in 10 liters of water, a small amount of thymol being used as a preservative.

The filtrates and washings from all of these preparations were analyzed. In two cases, as will be discussed later, the amount of nitrogen remaining in solution was so small that the analysis was carried out directly on the filtrate. The others were treated with tungstic acid to remove any remaining soluble protein and the filtrate from this treatment analyzed for non-protein nitrogen by a micro-Kjeldahl technique.

The moisture values were determined by drying samples to constant weight in the vacuum oven at 105° under a vacuum of 29 inches. The time required to attain this constancy varied between 8 and 78 hours. The amount of ash in the samples was found by incinerating aliquots at about 525° in 24 hour periods, until three successive weighings checked. The nitrogen figures were obtained by a modification of the Kjeldahl method suggested by Sørensen (5). The nitrogen values presented are calculated on a moisture- and ash-free basis.

Results

That there is a lowering in the nitrogen content of the crystalline proteins accompanying denaturation is shown in Figs. 1 to 5. These graphs were constructed by grouping the number of determinations in certain ranges and plotting each such range as abscissæ and a corresponding number of determinations as ordinates. Averages of the nitrogen determinations are given in Table I. All results are calculated on a moisture- and ash-free basis. That these differences are statistically significant can be further appreciated by a calculation of the *t* values, as described by Yule and Kendall (6). It may be said that a *t* value of 3 or greater shows a statistically significant difference. These values for the data given vary from 10.67 in the case of the edestin and edestan samples presented in Fig. 5 to 24.34 in the case of the crystalline albumin and its acid-denatured derivative (Fig. 3).

In order for the removal of a substance from the protein molecule to result in a diminution of its nitrogen content, it must necessarily be richer in nitrogen than the molecule as a whole. Ammonia is the nitrogenous material most likely to be split out under the ex-

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TABLE I
Comparison of Nitrogen Content of Native and Denatured Proteins

Native protein	Denatured protein	Amount of protein used in preparing denatured protein	Average N content of native protein	Total N in fil- trate from dena- turation	Non-protein N in filtrate from dena- turation	Average N found in denatured protein	Calculated N of de- natured protein*
		gm.	per cent	mg.	mg.	per cent	per cent
Edestin	Edestan	150	18.70		56	18.55	18.58
Crystalline Egg Albumin A	Alkali-denatured Egg Albumin A	76.9	15.44		10	15.20	15.21
Crystalline Egg Albumin B	Alkali-denatured Egg Albumin B	258.1	15.36		31	15.02	15.05†
" "	Acid-denatured Egg Albumin B	168.2	15.36	47		14.86	14.86
" "	Egg Albumin B denatured by shaking	168.2	15.36	117		14.88	14.95

* These results were obtained by assuming no loss of nitrogen during the process of denaturation.

† These results are really the composite of the analyses of two samples of alkali-denatured egg albumin prepared from crystalline Egg Albumin B. Since the results show that the two products are essentially identical, they have been listed together.

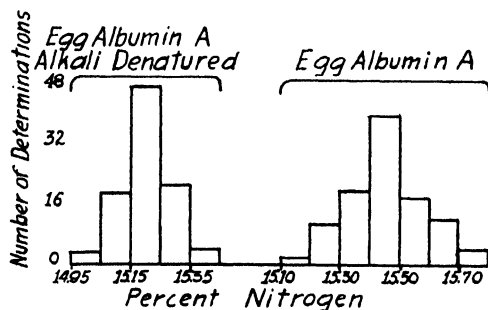


FIG. 1. Histograms comparing the frequency-distributions of the results of nitrogen determinations in crystalline Egg Albumin A (average N 15.44 per cent) and in alkali-denatured Egg Albumin A (average N, 15.20 per cent). Denaturation was induced by allowing the crystalline Egg Albumin A to stand in an excess of 0.1 N sodium hydroxide for 30 minutes.

perimental conditions employed in preparing the samples and could cause this effect. The loss of arginine or glycine would lead to the same result. In the calculations of Table I, we have assumed only nitrogen to be lost in the quantity shown. This is obviously not the case, but enables us easily to calculate the

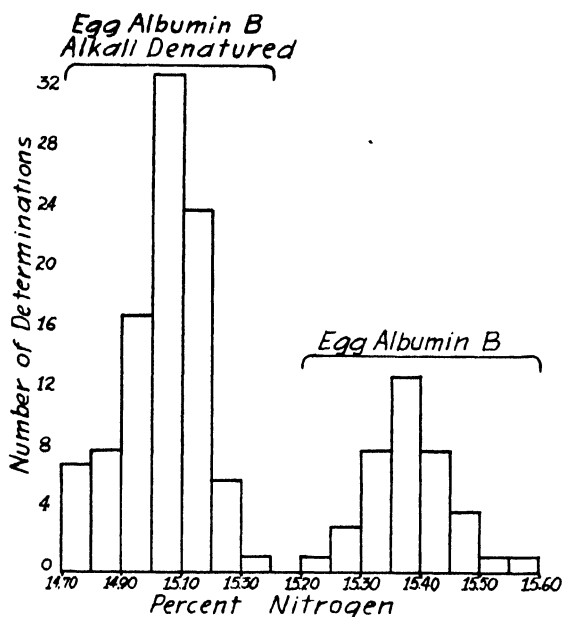


FIG. 2. Histograms comparing the frequency-distributions of the nitrogen determinations in crystalline Egg Albumin B (average N 15.36 per cent) and in alkali-denatured Egg Albumin B (average N 15.02 per cent). These determinations of nitrogen in the alkali-denatured Egg Albumin B were really made on two separate preparations but since the results show them to be identical they are grouped together here. Denaturation was induced by allowing the crystalline egg albumin to stand in an excess of 0.1 N sodium hydroxide for 6 hours.

maximum effect which the loss of such quantities of nitrogen could bring about. That the decrease in per cent nitrogen is not due to such losses from the protein can be seen from an examination of Table I. In the cases of the acid-denatured albumin shown in Fig. 3 and of the albumin denatured by shaking (Fig. 4), the treat-

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ment was such that only 47 and 117 mg. of nitrogen, respectively, remained in the filtrate. Since this nitrogen came from 168 gm. of albumin (moisture- and ash-free), it is easy to calculate that, had these derivatives not lost this amount of nitrogen, the product denatured by acid would have contained 14.88 per cent, and the albumin denatured by shaking, 14.95 per cent nitrogen. Comparison of these figures with the nitrogen content of 15.36 per cent for the crystalline material indicates that the amount of nitrogen in the protein has been lowered to an extent which cannot be

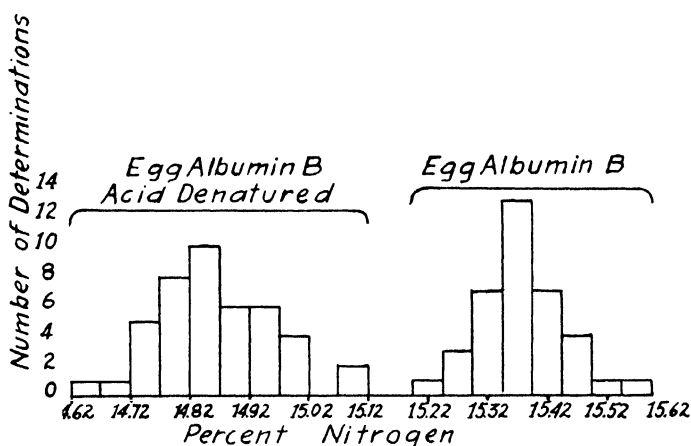


FIG. 3. Histograms comparing the frequency-distributions of the results of nitrogen determination in crystalline Egg Albumin B (average N 15.36 per cent) and acid-denatured Egg Albumin B (average N 14.86 per cent). Denaturation was induced by adjusting the solution to pH 1 with hydrochloric acid and allowing it to stand overnight.

accounted for by the splitting of nitrogenous materials from the molecule. Since an appreciable quantity of protein remained in solution after the other denaturation procedures, this was removed by precipitation with tungstic acid and the nitrogen in this filtrate determined. In no case was this non-protein nitrogen sufficient to account for the observed lowering.

Since the amount of nitrogen split off from the protein during the process of denaturation does not account for the decrease in the per cent of nitrogen, it is of interest to consider other ways by

which the nitrogen percentage might be decreased. An obvious suggestion is that a non-nitrogenous addition may be made to the protein molecule. In the methods which we used in denatura-

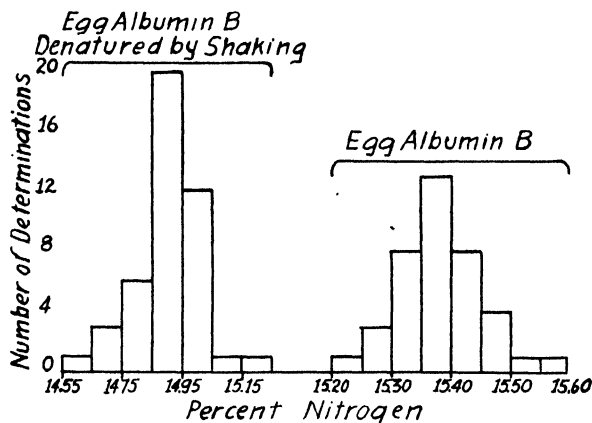


FIG. 4. Histograms comparing the frequency-distributions of the results of nitrogen determinations in crystalline Egg Albumin B (average N 15.36 per cent) and in Egg Albumin B denatured by shaking (average N 14.88 per cent). Denaturation was induced by shaking the solution of crystalline Egg Albumin B until precipitation was complete.

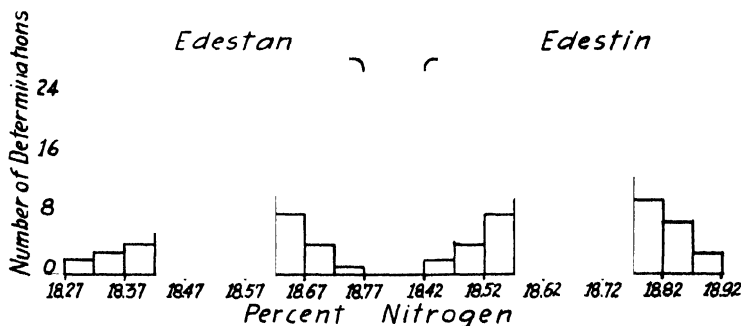


FIG. 5. Histograms comparing the frequency-distributions of the results of nitrogen determinations in edestin and edestan, average N 18.70 and 18.50 per cent, respectively.

tion, only water could have been added to the protein. Simple calculations show that in the case of one sample of edestin, 95 molecules of water would have to be added to produce the observed

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decrease in per cent of nitrogen, while in the case of one sample of egg albumin only 25 molecules of water need be added to a molecule of protein in order to bring about the change in nitrogen observed in egg albumin denatured by alkali. The amounts of water required in other cases of denaturation were between these two extremes. Such an addition of water might be brought about by hydrolysis without cleavage of the molecule. If the native protein is made up of a series of rings resembling diketopiperazine rings and then the rings are opened during the process of denaturation, water would be taken up and the percentage of nitrogen would decrease, as we have observed in our analyses.

SUMMARY

1. Denaturation of crystalline egg albumin by alkali, acid, or shaking results in a decrease in its percentage nitrogen.
2. Edestan contains less nitrogen per mole than does edestin.
3. The nitrogen removed from the protein during denaturation is insufficient to account for the observed effects.
4. It is suggested that these effects are explainable by a hydrolysis with direct addition of water to the protein molecule.

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A STUDY OF SULFHEMOGLOBIN*

By HARRY O. MICHEL

(From the Department of Biochemistry, Duke University School of Medicine,
Durham, North Carolina)

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The hemoglobin derivative produced by the action of hydrogen sulfide on oxyhemoglobin, now known as sulfhemoglobin, was first studied with the aid of the spectroscope by Hoppe-Seyler (1) who described the characteristic absorption band in the red region of the spectrum, slightly displaced toward the blue from the band for methemoglobin. The compound was named sulfmethemoglobin by Hoppe-Seyler, who found that it could not be formed in the absence of oxygen, nor could it be obtained in the crystalline state. Harnack (2) claimed to have prepared the compound in the absence of oxygen, but the experimental conditions described by him did not insure complete absence of air. Clarke and Hurlley (3) found that reducing agents such as sodium hydrosulfite or phenylhydrazine greatly catalyzed the formation of sulfhemoglobin from oxyhemoglobin and traces of hydrogen sulfide. They discovered also that carbon monoxide caused a shift in the absorption maximum of sulfhemoglobin of about 5 $m\mu$ toward the violet end of the spectrum. Haurowitz (4) attempted to prepare pure, crystalline sulfhemoglobin by treating horse oxyhemoglobin for a period of 1 week with hydrogen sulfide and air, but the analyses reported by him indicate that he did not obtain a pure product. Haurowitz did not believe that sulfhemoglobin bound carbon monoxide, but that the shift in the

* The data in this paper were taken from the thesis presented by Harry O. Michel to the Graduate School of Arts and Sciences of Duke University in May, 1938, in partial fulfilment of the requirements for the degree of Doctor of Philosophy. A preliminary report was given before the American Society of Biological Chemists at Baltimore, 1938 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. lxxxv (1938)).

absorption spectrum caused by this gas was due to the superimposed spectra occurring in the complex solution of pigments obtained by treating oxyhemoglobin with hydrogen sulfide. Keilin (5) showed definitely that sulfhemoglobin is not a methemoglobin compound by preparing a compound of methemoglobin with hydrogen sulfide, which gave a spectrum entirely different from that of sulfhemoglobin. Drabkin and Austin (6) were able to deduce the spectrum of sulfhemoglobin from solutions of oxyhemoglobin partially converted to sulfhemoglobin. A fairly complete recent review of the chemical and clinical work done on sulfhemoglobin is given by Giordano and Vigliani (7).

In view of the conflicting opinions in the literature with regard to the chemical mechanism of formation of sulfhemoglobin this problem was reinvestigated in general. In particular, experiments were designed in an attempt to establish the mechanism of formation and the stoichiometric relations of sulfur to iron in the sulfhemoglobin molecule, and to test the validity of the questionable carbon monoxide-sulfhemoglobin complex. This study also included further observations of the chemical and physical properties of sulfhemoglobin.

EXPERIMENTAL

The quantitative determination of sulfhemoglobin was carried out by use of the spectrophotometer, according to the technique suggested by Williamson (8), Heilmeyer (9), and Drabkin and Austin (10) and with the extinction coefficients for hemoglobin and sulfhemoglobin given by Drabkin and Austin (6). This was supplemented by gasometric and iron analyses. Further details on quantitative measurements and methods of preparing pigments are given in the thesis by the author (11).

Rôle of Peroxides in Sulfhemoglobin Formation

Crystalline dog hemoglobin was dissolved in 0.1 M phosphate buffer, pH 7.4, to a concentration of 0.2 per cent. To 5 cc. of this solution were added about 20 mg. of $\text{Na}_2\text{S}_2\text{O}_4$, after which only the absorption spectrum of reduced hemoglobin could be observed. To the solution was then added 1 drop of 0.1 M $(\text{NH}_4)_2\text{S}$. No change in the visible spectrum occurred. 1 drop of 3 per cent hydrogen peroxide was finally added, giving instantly

a strong sulfhemoglobin band at $620\text{ m}\mu$. This experiment could be repeated at will with either dog or human hemoglobin.

The above experiment was repeated with the substitution of 1 mg. of sodium perborate for hydrogen peroxide, giving the same result. The spectrum was observed continuously during the addition of sodium perborate, and at no time did the spectrum of oxyhemoglobin appear.

The effect of an old sample of benzoyl peroxide was tried and this caused the formation of a very weak sulfhemoglobin band.

Hemoglobin was reduced with excess sodium hydrosulfite and then 1 mg. of sodium perborate was added. No change in the spectrum occurred. A drop of dilute ammonium sulfide was added to the solution, with the spectrum continuing to remain unchanged. Finally to this solution was added another mg. of sodium perborate. Sulfhemoglobin formed at once.

To determine whether or not sodium hydrosulfite played a direct part in the peroxide effect, human hemoglobin in 0.2 per cent solution was deaerated in the Van Slyke manometric apparatus. A drop of 0.1 M $(\text{NH}_4)_2\text{S}$ was added to the reduced hemoglobin with precautions to exclude air. This did not cause any change in the spectrum. Sodium perborate, dissolved in air-free water, was then added, bringing about a rapid formation of sulfhemoglobin. Clarke and Hurtley (3) showed that although dilute solutions of oxyhemoglobin and H_2S form sulfhemoglobin very slowly, the addition of sodium hydrosulfite or phenylhydrazine caused the immediate formation of sulfhemoglobin. Van den Bergh and Wieringa (12) showed that no sulfhemoglobin is formed if sodium hydrosulfite is first added to oxyhemoglobin, followed by H_2S .

In an attempt to elucidate the $\text{Na}_2\text{S}_2\text{O}_4$ effect, the following experiments were performed. Human oxyhemoglobin, 0.2 per cent solution buffered at pH 7.0 with phosphate, was reduced with a large excess of $\text{Na}_2\text{S}_2\text{O}_4$. To 5 cc. of this solution were added 2 mg. of KCN. A drop of $(\text{NH}_4)_2\text{S}$, 0.1 M , produced no effect. The addition of 2 mg. of sodium perborate then produced sulfhemoglobin immediately. The experiment shows that potassium cyanide has no inhibiting effect on the formation of sulfhemoglobin.

To a dilute oxyhemoglobin solution, prepared as described in

the preceding experiment, were added a few mg. of KCN. Then the minimum quantity of $\text{Na}_2\text{S}_2\text{O}_4$ necessary to cause the disappearance of the oxyhemoglobin absorption bands was added. Immediately following this a drop of dilute ammonium sulfide was introduced into the solution, resulting in the formation of a weak but plain sulfhemoglobin band.

The above experiment was repeated with the omission of potassium cyanide. In this no sulfhemoglobin was formed.

From these experiments one may conclude that in the presence of cyanide, sodium hydrosulfite reacts with oxyhemoglobin to form hydrogen peroxide and hemoglobin. The subsequent addition of sulfide causes sulfhemoglobin to form. In the absence of cyanide, any peroxide which is formed is immediately decomposed by catalase, which is always present in hemoglobin solutions. Potassium cyanide poisons catalase and prevents the rapid destruction of peroxide.

The following experiments were performed in an attempt to find, from the amount of sulfhemoglobin formed and the amount of H_2S oxidized, just how much H_2S was used to form a given amount of sulfhemoglobin.

The oxygen uptake was measured in the Warburg respirometer. Buffered oxyhemoglobin solution was placed in the main vessel and the sulfide solution was added from the side arm. In general, sulfide solutions were freshly prepared by bubbling H_2S gas through 1 N or 0.1 N NaOH to the desired concentration. Fig. 1 shows the results of a typical experiment.

Preliminary experiments showed that the oxidation of H_2S in buffer solution alone in the region from pH 6 to 8 was very slow, increasing in rate with increase in pH. In the presence of oxyhemoglobin, the oxygen uptake is very rapid, the rate of oxidation increasing with hemoglobin concentration and with temperature. With smaller quantities of oxyhemoglobin, the final oxygen uptake is greater, owing to the fact that less sulfhemoglobin is formed, leaving more sulfide available for oxidation.

The total oxygen uptake shows that for every molecule of H_2S oxidized to sulfur 1 molecule of oxygen is used up.

In Fig. 1 is shown also the rate of formation of sulfhemoglobin plotted with the oxygen uptake curve. This experiment was performed by setting up a series of Warburg vessels, all containing

the same concentrations of oxyhemoglobin and sulfide. At the intervals noted, the contents of one Warburg vessel were removed and diluted 100 times with half saturated $(\text{NH}_4)_2\text{SO}_4$. This procedure reduces the rate of formation of more sulfhemoglobin to a negligible quantity, and also yields, after filtration, a clear solution which may be read in the spectrophotometer.

Effect of Various Oxidizing Agents on Sulfhemoglobin Formation

Having found that sulfhemoglobin can be formed from reduced hemoglobin and sulfide by adding peroxides, the effect of other oxidizing reagents was studied. Beef hemoglobin, 0.2 per cent

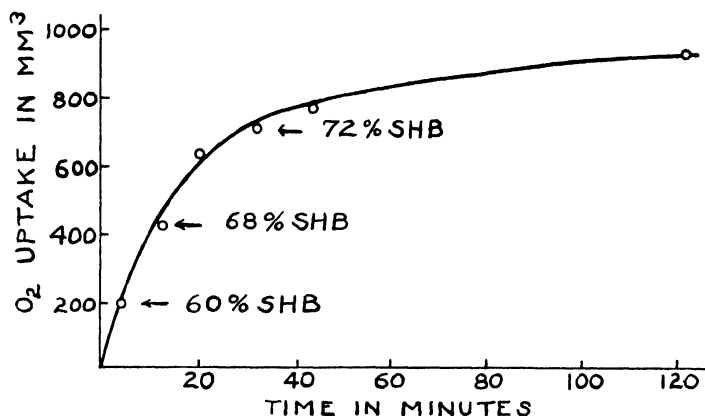


FIG. 1. Rates of O_2 uptake and SHb formation. The Warburg vessel contained 0.011 mm of HbO_2 and 0.042 mm of Na_2S . pH 7.4, 37° .

in phosphate buffer of pH 7.4, was extracted in the Van Slyke apparatus until the oxyhemoglobin absorption bands disappeared. Air-free 1 per cent potassium sulfide was added, 0.3 cc. being used. No change in spectrum occurred. Then 1 cc. of air-free 0.3 per cent potassium ferricyanide was added. No sulfhemoglobin formed. In the presence of sulfide, no methemoglobin band forms at $634 \text{ m}\mu$. After 2 hours there was still no sulfhemoglobin band visible. At this time, air was admitted, and sulfhemoglobin formed rapidly. The experiment was repeated, with air-free solutions of 8 per cent KClO_3 , 0.1 N KMnO_4 , 1 per cent NaClO , and 0.1 per cent *p*-aminophenol each in place of ferri-

cyanide, but in no case was sulfhemoglobin formed. Hemoglobin prepared as above was reduced with excess sodium hydro-sulfite and then dilute potassium sulfide was added. To this solution were added several drops of saturated bromine water. No sulfhemoglobin formed. Finally on addition of 1 mg. of sodium perborate, sulfhemoglobin formed at once. The experiment demonstrates that of a large number of oxidizing reagents, only hydrogen peroxide, or substances yielding this substance in water, will cause the formation of sulfhemoglobin from reduced hemoglobin and sulfide.

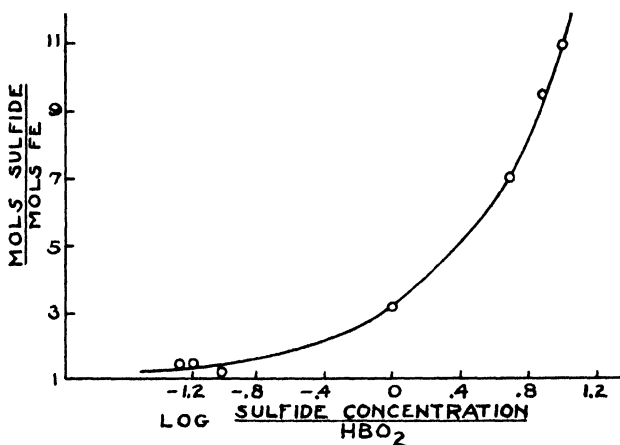


FIG. 2. Effect of sulfide concentration upon the fraction of Hb converted to SHb. $\text{HbO}_2 = 0.0118 \text{ M}$. pH 7.0, 25° .

Effect of Sulfide Concentration on Sulfhemoglobin Formation

In this experiment, varying amounts of sodium sulfide were added to a constant quantity of 0.012 M human oxyhemoglobin solution in a series of flasks. The solutions were buffered to pH 7.0, and the stoppered flasks were shaken slowly for approximately 30 minutes at 30° . The amount of sulfhemoglobin formed was then determined spectrophotometrically. The results are shown in Fig. 2. The log of the sulfide concentration is used so that the points for small concentrations would not be crowded together. The sulfide concentration, which was varied, is divided by the oxyhemoglobin concentration, which was kept

constant, so that the zero point on the abscissa of this curve represents an equimolecular solution of sulfide and oxyhemoglobin.

Along the ordinate is plotted the ratio of the moles of sulfide required to give 1 mole of sulfhemoglobin, 1 mole being assumed for each heme iron. Fig. 2 shows that at low sulfide concentrations a theoretical ratio of 1 atom of sulfur for each atom of sulfhemoglobin iron is approached. This is confirmed by the sulfur analyses as given in a subsequent part of this paper. With higher sulfide concentrations a greater ratio is indicated, owing to oxidation of a large part of the sulfide to elementary sulfur.

Effect of pH on Formation of Sulfhemoglobin

To 1 cc. portions of 8.1 per cent human oxyhemoglobin were added 0.5 cc. portions of McIlvaine's phosphate-citrate buffer, 1 M, at pH 6.0, 7.0, and 8.0. To each of the vessels was then added 0.5 cc. of 0.013 M K_2S , after which they were closed and shaken for 50 minutes at 37°. The solutions were then removed, diluted 100 times with half saturated ammonium sulfate, filtered, and read in the spectrophotometer. The solution at pH 6.0 formed 20 per cent sulfhemoglobin, that at pH 7.0 formed 12 per cent, and that at pH 8.0 formed 9 per cent. The experiment shows that other things being equal, the effect of increasing the pH is to decrease the amount of sulfhemoglobin formation.

Effects of Some Reagents on Sulfhemoglobin and Its Formation

A large number of qualitative experiments were carried out with sulfhemoglobin and on its formation in which most of the observations recorded in the literature were repeated, and some additional properties of the compound were noted.

In conformity with older observations, it was found that other forms of sulfur, such as those occurring in thiourea, cystine, cysteine, thioglycolic acid, sodium thiosulfate, sodium sulfite, and elementary sulfur, could not be substituted for sulfide sulfur in the formation of sulfhemoglobin. It was observed that 1 per cent formaldehyde did not prevent the formation of sulfhemoglobin and did not have any immediate action on it after formation. Lead acetate and iodoacetate, which react with the —SH group, were without appreciable action on sulfhemoglobin.

It was found that in the presence of a fairly large amount of

potassium cyanide, approximately 1 M, strongly buffered with phosphate to keep the pH below 8, oxidized sulfhemoglobin, upon being reduced with sodium hydrosulfite, gave rise to a new absorption band at 644 m μ , which could not be accounted for on the basis of any known hemoglobin derivative. When the solution was stirred, so that it became saturated with oxygen, the typical band of sulfhemoglobin at 620 m μ returned, replacing the band at 644 m μ . The compound is presumably a very unstable cyanide complex of reduced sulfhemoglobin, which only forms if a large amount of cyanide is present and easily reverts to sulfhemoglobin.

In general these experiments present evidence that sulfhemoglobin can be formed only from soluble sulfides as the source of sulfur, and that the compound formed is not a sulfhydryl type, or at least not like any known sulfhydryl compound.

Stability of Oxidized and Reduced Sulfhemoglobin

If a solution containing a mixture of sulfhemoglobin and oxyhemoglobin is permitted to stand in contact with air, at pH 7.0, either at 0° or room temperature, a brownish precipitate forms gradually. In the course of a week at room temperature, two-thirds of the pigment may be precipitated. The precipitate dissolves in dilute ammonium hydroxide and gives a typical denatured globin hemochromogen spectrum on reduction with sodium hydrosulfite. The iron content of the precipitate was found to be 0.30 per cent.

In contrast to the fairly rapid decomposition of sulfhemoglobin in contact with oxygen, reduced sulfhemoglobin is very stable. A solution of human hemoglobin containing 60 per cent of the total pigment as sulfhemoglobin at pH 7.0 was kept reduced with excess sodium hydrosulfite and left at room temperature. After 3 months there was no apparent decrease in the intensity of the sulfhemoglobin absorption band. This is in conformity with the clinical observations of the extreme stability of sulfhemoglobin in the blood as contrasted with other abnormal blood pigments.

Alkaline Denaturation of Sulfhemoglobin

In this experiment a study was made of the stabilities of the hemoglobins from two different species and of their corresponding

sulfhemoglobins, 0.05 N NaOH being used as a denaturing agent. In the usual method of measuring the rate of alkaline denaturation, as used extensively by Haurowitz (4), the oxygenated form of hemoglobin is used. The reaction proceeds in two steps, reduced hemochromogen being first formed, followed by a rapid oxidation to the oxidized form of the hemochromogen by the oxygen present in the solution. To avoid the necessity of measuring two simultaneously proceeding reactions, all solutions were reduced with excess sodium hydrosulfite. When reduced hemochromogen is formed, an intense absorption band appears at

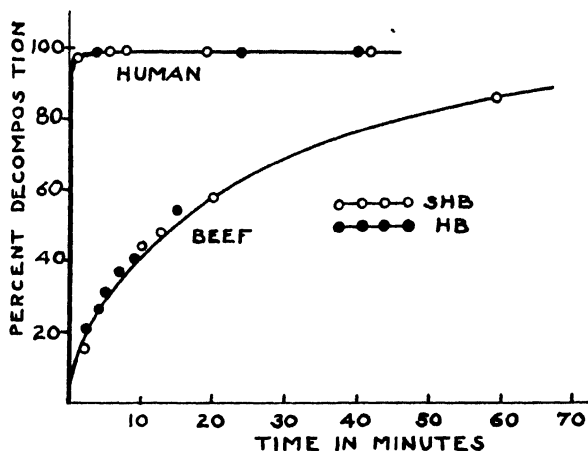


FIG. 3. Rate of denaturation of human and beef Hb and SHb in 0.05 N NaOH, 25°.

558.6 $m\mu$. By measuring the rate of increase of light absorption in the reduced alkaline hemoglobin solution with a spectrophotometer, a measure of the rate of denaturation of hemoglobin was obtained. A similar procedure has been employed by Drabkin and Austin (13), who used dog hemoglobin and a lower alkalinity than employed here.

Since sulfhemoglobin in alkaline solutions is converted to denatured globin hemochromogen, with the disappearance of the characteristic absorption band at 620 $m\mu$, a measure of the rate of denaturation of sulfhemoglobin was obtained by determining the decrease in light absorption at 620 $m\mu$ with the spectrophotometer.

The rates of denaturation of human and beef hemoglobin and of their corresponding sulfhemoglobins in 0.05 N NaOH are shown in Fig. 3. The percentage decomposition was calculated on the basis of the fractions of the initial or final absorption remaining at any time. The results show that the resistance to alkaline denaturation of sulfhemoglobin is practically identical to that of the hemoglobin from which it was derived.

Sulfhemoglobin from Hematoporphyrin

In considering the nature of sulfhemoglobin, it is important to know whether the formation of this compound is a special property of protoporphyrin, the natural porphyrin occurring in hemoglobin, or whether it can be formed from artificial hemoglobins containing other porphyrins.

The most readily obtainable porphyrin is hematoporphyrin, which never occurs naturally but may be easily obtained by the action of concentrated sulfuric acid on hemoglobin. The method described by Kaplan (14) was used for the preparation of hematoporphyrin.

Hill (15) has shown that an iron derivative of hematoporphyrin can be prepared, and Hill and Holden (16) have shown that the compound so produced will combine with native globin.

Hematoheme was prepared by heating hematoporphyrin with iron filings in glacial acetic acid for 4 hours on the steam bath and then precipitating the compound by dilution with water. To be certain that a heme compound had been formed, the pyridine hemochromogen was prepared by dissolving hematoheme in 40 per cent pyridine and reducing with sodium hydrosulfite. A typical hemochromogen spectrum was obtained, with bands at 548 and 519 $m\mu$.

Hematoheme was dissolved in dilute sodium carbonate and then added to native beef globin, buffered to pH 7.4 with phosphate buffer. The globin was prepared according to the method of Roche and Combette (17). It was found very difficult to prepare any appreciable amount of native globin from human or dog blood, but beef blood gave good yields.

The hematohemoglobin, on being reduced by sodium hydrosulfite, gave a single broad absorption band, very similar to re-

duced hemoglobin. After oxygenation of the solution, two bands appeared, similar to oxyhemoglobin, at 573 and 537 $m\mu$. Upon addition of a few drops of saturated H_2S water, and reduction with sodium hydrosulfite, an absorption band formed at 618 $m\mu$. The band was very poorly defined, but had the properties of a sulfhemoglobin band, being destroyed by alkali. The denatured globin hemochromogen from hematoporphyrin which formed in 0.1 N NaOH had absorption bands at 552 and 522 $m\mu$. The corresponding bands for ordinary denatured globin hemochromogen are at 558 and 528 $m\mu$.

On the basis of these results, it may be concluded that protoporphyrin possesses no special structure which is essential to the formation of sulfhemoglobin, and also that sulfhemoglobins may be prepared from reconstituted hemoglobins.

Molecular Weight of Sulfhemoglobin

The molecular weight of sulfhemoglobin was estimated by means of its osmotic pressure. An approximately 3 per cent solution of human oxyhemoglobin buffered to pH 6.7 gave an osmotic pressure by means of an osmometer corresponding to a molecular weight of 68,000. After conversion of the oxyhemoglobin to 83 per cent sulfhemoglobin, the average molecular weight was 66,000, indicating that no significant aggregation or dissociation occurs when oxyhemoglobin is transformed to sulfhemoglobin.

Solubility of Sulfhemoglobin

Sulfhemoglobinemia was produced in rats by mixing powdered sulfur and phenacetin with their food. To twelve rats, averaging 75 gm. in weight, were given 3 gm. of sulfur and 1 gm. of phenacetin daily. After 5 days, one of the animals was killed and the blood diluted with 1 volume of water. To the diluted blood was added $\frac{1}{4}$ volume of toluene, and the mixture was thoroughly shaken and placed in the refrigerator to allow crystallization to occur.

The crystals and mother liquor were separated by centrifugation, and, after the crystals were dissolved with the aid of sodium carbonate, both solutions were analyzed with the spectrophotometer. By means of the hand spectroscopic, a very plain sulfhemoglobin absorption band could be seen.

In the crystals was found 5 per cent SHb and in the supernatant, 4 per cent, showing, within the experimental error, that the solubility of the sulfhemoglobin and oxyhemoglobin was approximately the same. The sulfhemoglobin-containing crystals were examined under the high power field of a microscope. The crystals were sharply defined, 4- and 5-sided, thin plates, all of which appeared to be of a single type indistinguishable from the normal hemoglobin crystals, making it unlikely that sulfhemoglobin had formed in separate crystals from oxyhemoglobin, but rather a solid solution had formed.

Washed dog red blood cells were treated with H_2S , washed again with physiological saline, and then hemolyzed by mixing with $\frac{1}{2}$ volume of toluene. After the mixture had stood in the cold for 24 hours, the crystals which had formed in very small amount were separated from the supernatant fluid, dissolved with the minimum quantity of sodium carbonate, and analyzed spectrophotometrically, as was the supernatant fluid. In the crystals 23 per cent and in the supernatant 22 per cent sulfhemoglobin was found.

Sulfhemoglobinemia was produced in a dog by giving daily doses of 1 gm. of powdered sulfur and 1 gm. of phenacetin mixed with ground beef. Crystals and supernatant fluid were obtained from the blood of this animal after 30 days of the above treatment, the procedure described above for rat blood being followed. Spectrophotometric analysis showed 8.5 per cent sulfhemoglobin in the crystals and 9 per cent in the supernatant fluid.

As the solubilities of rat and dog oxyhemoglobins were found to be the same as the corresponding sulfhemoglobins, an attempt to separate dog sulfhemoglobin from reduced hemoglobin was made, since it is known that reduced dog hemoglobin is very much more soluble than the oxyhemoglobin from the same species.

Crystalline dog oxyhemoglobin was converted partially to sulfhemoglobin (40 per cent) in the usual way. The total pigment concentration was 14 gm. per cent. Phosphate buffer, pH 6.7, was added to a concentration of 0.1 M. The solution was deaerated with an oil pump, and was left under nitrogen at 2°. No crystallization occurred during a period of 1 week.

The separation of sulfhemoglobin from oxyhemoglobin was attempted by means of the salting-out procedure. Human oxyhemoglobin containing 70 per cent sulfhemoglobin was half

saturated with ammonium sulfate and left overnight in the refrigerator. The salted-out protein and supernatant were separated by filtration and analyzed spectrophotometrically. The percentage of sulfhemoglobin in the total pigment of the solid and liquid phases was the same.

The relative solubilities of reduced human hemoglobin and sulfhemoglobin were examined in strong phosphate buffer at pH 6.7. Reduction was brought about by addition of excess $\text{Na}_2\text{S}_2\text{O}_4$. Phosphate buffer, 2.8 M, was added until precipitation commenced. The mixture was left for 8 hours at 25° in a stoppered centrifuge tube. After centrifugation the ratio of sulf-

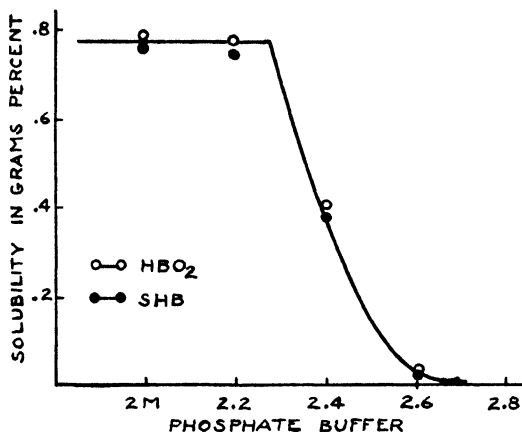


FIG. 4. Solubilities of beef HbO_2 and SHb in phosphate buffer, pH 7.0, 25°. The concentration of SHb was 67 per cent of the total pigment.

hemoglobin to total pigment in the supernatant fluid and precipitate was found to be the same.

The solubility of beef sulfhemoglobin and oxyhemoglobin was investigated at different concentrations of phosphate buffer. The results are shown in Fig. 4. These results show that under the conditions employed hemoglobin and sulfhemoglobin do not differ measurably with regard to their solubilities.

Formation of CO-Sulfhemoglobin

The formation of a carbon monoxide compound of sulfhemoglobin was first suggested by Clarke and Hurtley (3) who discovered that passing CO gas into solutions containing sulf-

hemoglobin caused a shift in the characteristic absorption band at $620\text{ m}\mu$, of approximately 5 to $7\text{ m}\mu$, toward the violet end of the spectrum. On the basis of experiments performed by him, Haurowitz (4) denied the possible existence of a compound between CO and sulfhemoglobin.

The shift in the spectrum of sulfhemoglobin which occurs when CO is passed into the solution of the pigment is shown in Fig. 5. The formation of a new compound is indicated by this shift, since it is not possible to construct any mixture of pigments which might

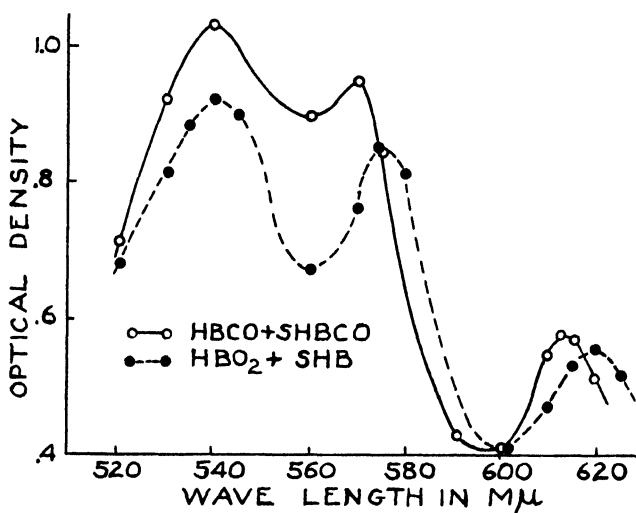


FIG. 5. Effect of CO on the absorption of SHb. The SHb represented 64 per cent of the total pigment, pH 7.0.

be present and would give the observed effect. The same shift in the spectrum is observed when mixtures of oxyhemoglobin and sulfhemoglobin are completely reduced with $\text{Na}_2\text{S}_2\text{O}_4$ before addition of CO gas.

Since no evidence of a gasometric study of the reaction between sulfhemoglobin and carbon monoxide could be found in the literature, the reaction was studied gasometrically, with the Van Slyke manometric apparatus and the Warburg respirometer.

In the Warburg procedure, sulfhemoglobin was freshly prepared from oxyhemoglobin which was mixed with sulfide in the closed

vessel and shaken until the oxygen uptake came to a stop. The vessels were then opened and acid ferricyanide was added to the side arm and 40 per cent KOH to the central inset vessel. The Warburg vessels were then saturated with air in those cases in which the oxygen capacity was being determined, and with CO for the carbon monoxide capacity. The addition of acid ferricyanide from the side arm releases oxygen from oxyhemoglobin and carbon monoxide from its combinations with hemoglobin and sulfhemoglobin and the amount of gas formed was determined manometrically.

TABLE I
Combination of SHb with Carbon Monoxide

Original Hb binding CO or O ₂	After partial conversion to SHb			
	SHb, spectropho- metric	Binding O ₂	Binding CO*	Extinction coefficient of globin hemo- chromogen, original Hb = 1.93
<i>gm. per cent</i>	<i>per cent</i>	<i>gm. per cent</i>	<i>gm. per cent</i>	
15.9	68	6.0	12.2	1.55
18.1	51	8.2	16.0	
17.7	47	6.8	15.6	
16.0	88		12.3	
18.2	87		10.8	
	75			1.35
	30			1.86

* Represents total intact Hb and SHb.

The results of these gasometric analyses are shown in Table I. The hemochromogen absorption coefficients shown in Table I give a measure of the total amount of heme pigment left after oxyhemoglobin is partially converted to sulfhemoglobin. The absorption coefficient as used in this paper is defined as the negative log of the fraction of light transmitted by a layer of solution 1 cm. in thickness and containing 0.1 per cent pigment.

The possibility existed that the extra carbon monoxide capacity of the sulfhemoglobin solutions examined was due to hemochromogen which might have formed during the preparation of sulfhemoglobin. However, at no time was there any spectroscopic evidence for the presence of hemochromogen in freshly prepared

solutions of sulfhemoglobin, after reduction with sodium hydrosulfite. Owing to the great intensity and sharpness of the spectrum of reduced hemochromogen, it is possible to detect very small amounts of this pigment in the presence of other pigments.

In the absence of active reducing agents, such as $\text{Na}_2\text{S}_2\text{O}_4$, or alkaline sulfides, hemochromogen is oxidized by air practically instantaneously to its ferric state, in which form it does not bind CO. In the experiments with the Warburg respirometer no CO was added to the oxygenated solution of sulfhemoglobin until all the sulfide had been oxidized, so that any hemochromogen which might have been present would have been in the oxidized form and so incapable of combination with CO.

The results indicate that probably 1 molecule of CO is bound for every atom of iron in reduced sulfhemoglobin.

Cataphoresis of Sulfhemoglobin and Oxyhemoglobin

The cataphoresis of sulfhemoglobin and oxyhemoglobin was carried out in a U-tube type of apparatus, with reversible copper-copper sulfate electrodes, according to the procedure described by Michaelis (18). An oxyhemoglobin solution containing 1.49 gm. per cent total pigment, of which 81 per cent was sulfhemoglobin, was buffered to various pH values with 0.05 M phosphate buffer. At pH 6.8 no movement of pigment occurred in 4 hours. At pH 7.8 the pigment moved 3 cm. toward the anode in 6 hours.

The pigment column was separated into four parts, Part 1 being nearest the anode and Part 4 nearest the cathode. The ratio of sulfhemoglobin to total pigment was determined in each section by means of the spectrophotometer. The results were as follows: Part 1, 82 per cent of the total was sulfhemoglobin; Part 2, 84 per cent; Part 3, 80 per cent; Part 4, 83 per cent.

From these results it is deduced that the isoelectric points and electrophoretic mobilities of sulfhemoglobin and oxyhemoglobin are not significantly different.

Sulfur Content of Sulfhemoglobin

The only quantitative data on the sulfur content of sulfhemoglobin are those reported by Haurowitz (4). The values obtained were very variable and quite high, making it appear unlikely that Haurowitz was working with a pure compound.

In various attempts to purify sulfhemoglobin it was found very difficult to separate extraneous sulfur by extraction or precipitation methods, so that excessively high sulfur values were obtained, similar to those found by Haurowitz. It was finally decided that cataphoresis offered the best possibility of accomplishing the purification of sulfhemoglobin.

Good descriptions of the cataphoretic method are given in the works of Tiselius (19) and Theorell (20). However, in working with sulfhemoglobin a sufficient quantity of material must be obtained in the shortest time possible because of the instability of the compound in oxygenated solutions. The methods already described did not meet these requirements, so a procedure worked out by Dr. T. B. Coolidge¹ of this laboratory was tried out and found to be suitable. With this apparatus a yield of over 1 gm. of total pigment could be obtained in about 12 hours.

In order to dry the purified sulfhemoglobin with a minimum amount of denaturation the solution was placed in a porcelain dish, and a stream of air, which had been passed over calcium chloride, was blown over the surface. After 12 hours the pigment was sufficiently dried so that it could be ground to a fine powder in a mortar. Further drying was carried out in a desiccator over sulfuric acid.

The sulfur was determined by fusing the sample in a micro-Parr bomb, precipitating the sulfate formed with barium, and weighing on the microbalance.

The following averaged figures for total sulfur were obtained.

	Per cent S
Human HbO ₂	0.614 (0.601-0.631)
“ SHb 38% + HbO ₂ 62%	0.700 (0.685-0.711)
“ “ 45% + “ 55%	0.717 (0.710-0.725)
Extra sulfur due to sulfhemoglobin	0.228
1 sulfur atom per sulfhemoglobin iron atom requires	0.192% S

Labile Sulfur Split from Sulfhemoglobin by Bromine

Hemoglobin solutions partially converted to sulfhemoglobin, and purified by cataphoresis, were saturated with bromine vapor, according to the method of Blumenthal and Clarke (21). After standing an hour, protein was precipitated with 20 per cent tri-

¹ Coolidge, T. B., personal communication, to be published.

chloroacetic acid, and the solutions were filtered and analyzed for sulfate gravimetrically. By this treatment only a negligible quantity of sulfate was obtained from oxyhemoglobin.

The labile sulfur found in three different samples is shown.

Human SHb, per cent of total pigment	Per cent S
16	0.238 (0.220-0.255)
42	0.272 (0.267-0.277)
28	0.264 (0.254-0.281)
Average amount of labile S.....	0.258
Expected, on basis of Fe:S = 1.....	0.192

From these results and from the analyses of the total sulfur in sulfhemoglobin it may be concluded that in the formation of sulfhemoglobin 1 atom of sulfur is introduced into the hemoglobin molecule for each iron atom, in a state which is easily oxidized to sulfate by bromine.

Myosulfhemoglobin

Beef muscle hemoglobin or myohemoglobin was prepared from fresh, lean beef according to the method described by Theorell (22) for beef heart. During the final stages of purification the myohemoglobin rapidly oxidized to the ferric form and was thus preserved and used in this state.

When dilute hydrogen sulfide solution was added to a solution of myohemoglobin at pH 7.2 and followed by a little sodium hydrosulfite, myosulfhemoglobin formed at once, as indicated by the appearance of a strong absorption band at 617 to 618 $m\mu$.

The addition of dilute potassium ferricyanide to myosulfhemoglobin caused the instant disappearance of the band at 617 $m\mu$. Then the addition of a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$ brought the band back at once to its original intensity.

Owing to the rapid and reversible oxidation of myosulfhemoglobin, it was found possible to determine the approximate number of equivalents of oxidizing reagent necessary to convert sulfhemoglobin to its ferric form.

The solution of myosulfhemoglobin used in this experiment contained a small amount of oxymyohemoglobin, but this is readily oxidized by potassium ferricyanide, so that it does not interfere with the interpretation of the results.

The total pigment concentration was determined by converting

all the hemoglobin derivatives to pyridine hemochromogen and reading in the spectrophotometer at $\lambda = 525 \text{ m}\mu$, according to the procedure described by Drabkin and Austin (6). More recently, Drabkin (23) has applied this procedure to the determination of total pigment in mixtures containing sulfhemoglobin. The concentration of pigment was 0.498 per cent or 0.30 mm.

The myosulfhemoglobin solution was titrated with 0.01 M $\text{K}_3\text{Fe}(\text{CN})_6$, the end-point in the reaction being determined as that point at which further addition of ferricyanide caused no further decrease in absorption at $617 \text{ m}\mu$. For 6.15 cc. of sulfhemoglobin solution was required 0.15 cc. of 0.01 M $\text{K}_3\text{Fe}(\text{CN})_6$.

The absorption coefficient of the myosulfhemoglobin solution at the start of the titration was $E_{617} = 4.03$ and at the end $E_{617} = 1.38$. At the end of the titration excess sodium hydrosulfite was added to the solution to convert completely the oxidized myosulfhemoglobin back to the reduced form. The absorption coefficient was then found to be $E_{617} = 4.40$, indicating that the myosulfhemoglobin was partially oxidized by atmospheric oxygen at the beginning of the titration. The titration result is corrected by the factor $(4.40 - 1.38)/(4.03 - 1.38)$, giving the corrected titration value of 0.17 cc. of 0.01 M $\text{K}_3\text{Fe}(\text{CN})_6$. Theoretically to oxidize 6.5 cc. of 0.30 mm sulfhemoglobin, assuming a ferrous-ferric couple, there would be required 0.195 cc. of 0.01 M $\text{K}_3\text{Fe}(\text{CN})_6$, which is sufficiently close to the experimental value.

In agreement with Theorell (22) it was found that beef myohemoglobin is extremely stable toward alkali, being slowly denatured over the course of 24 hours in 0.1 N NaOH. The interesting observation was made that myosulfhemoglobin was equally stable toward alkaline denaturation, and led to a study of the relative resistances of blood hemoglobin and sulfhemoglobin to the action of alkali, described above.

The action of 1 per cent HgCl_2 was tested on a solution of myosulfhemoglobin. A few drops of mercury solution caused the band at $617 \text{ m}\mu$ to disappear. On addition of sodium hydrosulfite to the solution the band at $617 \text{ m}\mu$ returned, suggesting that the action of mercuric chloride is one of oxidation. No formation of hemochromogen occurred, such as one obtains in the corresponding experiment with sulfhemoglobin from blood.

The spectrum of myosulfhemoglobin is shown in Fig. 6.

The data obtained in this experiment indicate that a very stable myosulfhemoglobin can be formed and that the resulting compound can be reversibly oxidized. The spectrum shows that the absorption maximum of myosulfhemoglobin is shifted slightly toward the blue end of the spectrum as compared with that of blood sulfhemoglobin and is almost twice as intense. The approximate specific absorption coefficient of myosulfhemoglobin is $E_{617} = 1.03$ and for blood sulfhemoglobin $E_{620} = 0.64$. The broken line curve in Fig. 6 shows the effect produced by CO on

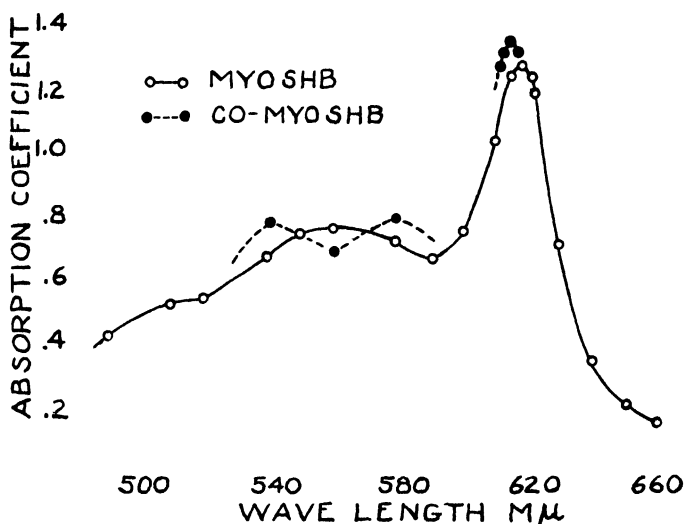


FIG. 6. Spectra of myosulfhemoglobin and its CO compound. The concentration of the total pigment was 1.36 gm. per cent, pH 7.0.

the spectrum of myosulfhemoglobin. It can be seen that CO causes a slight shift toward the blue in the maximum at 617 $m\mu$. The two new maxima at 540 and 579 $m\mu$ indicate the slight contamination of myosulfhemoglobin with myohemoglobin.

DISCUSSION

From the results shown in Fig. 2 it can be seen that sulfhemoglobin involves 1 atom of sulfide sulfur for each Fe atom of sulfhemoglobin produced. Fig. 2 shows that when a large excess of sulfide is used, most of it is oxidized. With a molar ratio of sul-

fide to oxyhemoglobin of 1, only one-third of the theoretically possible sulfhemoglobin is formed. By reducing the sulfide concentration to a low value the theoretical ratio is approached but never attained, owing to the fact that there is always a small fraction of the sulfide oxidized and also because it is experimentally impossible to determine with any accuracy the very small amounts of sulfhemoglobin which are formed.

The experiments in which sulfate was formed on treating sulfhemoglobin with bromine confirm the results described in the preceding paragraph. It was found that hemoglobin does not give sulfate on being treated with bromine. This was to be expected since Blumenthal and Clarke (21) have shown that cystine, cysteine, and methionine, which apparently account for all the sulfur in hemoglobin, do not yield sulfate on bromine oxidation. These workers found, however, that such compounds as thiourea, thioamides, and thiolhistidine do give sulfate when oxidized with bromine. It is not to be expected that any stable mercaptan type of bonding would occur upon treating a solution of oxyhemoglobin with sulfide at room temperature, but rather a relatively unstable type of linkage which would be susceptible to bromine oxidation such as was found experimentally. The formation of methylene blue, which involves the linking of 2 molecules of *p*-aminodimethylaniline by hydrogen sulfide in the presence of ferric chloride to form a thiazine ring, is a well known type of reaction to which the reaction to form sulfhemoglobin might be related.

It is less reliable to draw conclusions from the results of the total sulfur content in hemoglobin before and after partial conversion to sulfhemoglobin, the theoretical increase in the sulfur content being in these experiments of the order of less than 0.1 per cent. However, it was found that in the purest samples of sulfhemoglobin obtained by cataphoresis, the increase in sulfur content due to the formation of sulfhemoglobin corresponded closely to 1 sulfur atom for each atom of iron.

With few exceptions the workers in this field have shown that sulfhemoglobin can be formed from hemoglobin and sulfide only in the presence of oxygen. Clarke and Hurtley (3) observed that the addition of a reducing agent to oxyhemoglobin and a trace of sulfide greatly catalyze the formation of sulfhemoglobin. This apparently anomalous result, which has been con-

firmed by other workers, and also in these experiments, seems inexplicable at first sight, because it leads to the conclusion that the formation of reduced hemoglobin speeds the formation of sulfhemoglobin, whereas it has been definitely shown that sulfide has no action on reduced hemoglobin.

All the known facts regarding the formation of sulfhemoglobin are correlated by introducing hydrogen peroxide as a necessary component in the reaction. The most active catalyst for the formation of sulfhemoglobin from oxyhemoglobin and sulfide is sodium hydrosulfite. This compound also reacts faster with molecular oxygen to form hydrogen peroxide as one of its products than most other reducing agents, as stated by Mellor (24) and others. Phenylhydrazine also reacts quite rapidly with oxygen, very probably forming hydrogen peroxide as a product. It also catalyzes the formation of sulfhemoglobin.

The data on the oxidation of sulfide by oxygen in the presence of hemoglobin indicate the involvement of hydrogen peroxide. The oxygen uptake, when relatively small amounts of oxyhemoglobin were used, corresponded to 1 mole of oxygen for each mole of sulfide used. This corresponds to the oxidation of the sulfide to elementary sulfur with the formation of hydrogen peroxide. In the absence of hemoglobin the oxidation of hydrogen sulfide to sulfur is very slow. The oxidation of hydrogen sulfide by oxyhemoglobin and the subsequent formation of sulfhemoglobin can be represented by the following equations.



Only a small part of the hydrogen peroxide can conceivably be utilized in the formation of sulfhemoglobin. The remainder must be dissipated by directly attacking the globin or heme of hemoglobin. That this can occur with hemoglobin has been shown by Keilin (25) and Bingold (26) if the catalase is destroyed by heat or inhibited by cyanide or sulfide ion. Evidence for the destruction of heme pigment on conversion of hemoglobin to sulfhemoglobin is shown in Table I. The total pigment which was convertible to denatured globin hemochromogen had been decreased by 4 per cent in a solution containing 30 per cent sulfhemoglobin,

but in a solution containing 75 per cent of the total pigment as sulfhemoglobin formed by using a large excess of H_2S and thus presumably involving a large amount of hydrogen peroxide, the destruction of heme pigment amounted to 30 per cent.

In sulfhemoglobinemia, there is a great deal of evidence indicating that acetanilide or related derivatives are very important or necessary factors along with an intestinal condition which is conducive to the production of hydrogen sulfide. In the absence of these drugs, or similar factors, hydrogen sulfide rarely, if ever, has been shown to produce sulfhemoglobin in the intact animal. It has been shown by Michel, Bernheim, and Bernheim (27) that *p*-aminophenol is readily produced in tissues from acetanilide. *In vitro* acetanilide or aniline does not catalyze the formation of sulfhemoglobin from oxyhemoglobin and sulfide. Ivens and van Vollenhoven (28) have shown that *p*-aminophenol does bring about this catalysis. Since *p*-aminophenol is fairly rapidly autoxidizable to a quinone form with the probable formation of hydrogen peroxide as a result of its reaction with oxygen, it is very likely that it catalyzes the formation of sulfhemoglobin by causing the production of hydrogen peroxide.

The formation of sulfhemoglobin does not involve any detectable change in the globin, or protein part of the hemoglobin molecule. The rates of alkaline denaturation of human and beef hemoglobin, which differ greatly, remain unchanged on conversion of these compounds to their corresponding sulfhemoglobins. Since it is the globin part of the hemoglobin molecule which determines the alkaline denaturation rate, these experiments indicate that apparently no important change in the globin has occurred.

From the solubility measurements it can be seen also that no major change in the hemoglobin molecule takes place upon conversion to sulfhemoglobin.

The cataphoretic measurements and the molecular weight determination show that no aggregation or dissociation has occurred.

Apparently no special porphyrin is necessary for the formation of sulfhemoglobin, since hemoglobins prepared from protoporphyrin and hematoporphyrin both gave absorption bands in the region of 620 $m\mu$, having the properties ascribed to sulfhemoglobin.

In forming sulfhemoglobin from hemoglobin there is no irrevers-

ible change in the heme. This is shown by the fact that hemochromogens may be prepared from sulfhemoglobin which do not appear to differ from the corresponding compounds prepared from hemoglobin. List (29) showed that the porphyrin obtained from sulfhemoglobin was spectroscopically identical with protoporphyrin obtained from hemin or hemoglobin.

The sulfhemoglobin derivative of myohemoglobin was prepared very easily, giving an absorption band at $618\text{ m}\mu$ which had almost double the intensity of the corresponding band in blood sulfhemoglobin. Theorell (20) has pointed out that the molecular weight of muscle hemoglobin is only half that of blood hemoglobin, so that each molecule contains only two heme groups, the iron content being the same as in blood hemoglobin. The formation of mysulfhemoglobin indicates that the four-heme structure possessed by blood hemoglobin is not a necessary factor in the formation of sulfhemoglobin.

One of the interesting difficulties encountered in working with sulfhemoglobin was the failure to convert completely hemoglobin to sulfhemoglobin when the compound was treated with an excess of sulfide. This fact seems to indicate that an equilibrium is reached. However, this is unlikely, because it is impossible to reconvert sulfhemoglobin to hemoglobin either by exposing it to a vacuum for several days or by prolonged dialysis. The answer apparently resides in the complexity of the reaction involving hydrogen peroxide. In order to get a good yield of sulfhemoglobin just the proper amount of hydrogen peroxide must be formed, and most of this must presumably be used in forming sulfhemoglobin. If an excessive amount of peroxide is formed, it will directly attack the pigment, causing some heme destruction. Also it will oxidize hemoglobin to methemoglobin, with which it combines to form a very slightly dissociated compound having a spectrum qualitatively similar to that of oxyhemoglobin.

From the major change which occurs in the visible spectrum on conversion of oxyhemoglobin to sulfhemoglobin it seems that the heme iron must be involved. Major changes in the globin, involving marked differences in composition and in resistance to denaturation, as shown by human and beef hemoglobin, do not cause any detectable differences in the absorption spectra of the oxyhemoglobin compounds. However, any change involving the

heme iron in any hemoglobin compound results in a marked change in the absorption spectrum, such as the change from the reduced to the oxygenated form.

The formation of a carbon monoxide compound of sulfhemoglobin indicates that the sulfur cannot occupy the place normally occupied by oxygen or carbon monoxide. The gasometric determinations of the carbon monoxide capacity of sulfhemoglobin indicate that slightly less than 1 mole of carbon monoxide is found for each iron atom, but this can be correlated with the fact that pigment destruction had occurred, as shown by the absorption coefficients of the denatured globin hemochromogens from the corresponding sulfhemoglobin solutions being diminished below that for the original hemoglobin.

From the evidence it seems possible that the formation of sulfhemoglobin may involve the globin heme linkage, which has never been definitely explained but is apparently through the iron. The instability of the oxidized form of sulfhemoglobin would probably support this hypothesis. A clearer insight to this question will probably have to involve newer approaches, such as the magnetic method used by Coryell, Stitt, and Pauling (30) in working out the structures of hemoglobin and some of its derivatives.

SUMMARY

1. Sulfhemoglobin is formed from hemoglobin and in the presence of any soluble inorganic sulfide by the action of hydrogen peroxide.

2. Hydrogen sulfide is catalytically oxidized by oxygen in the presence of hemoglobin to sulfur and hydrogen peroxide.

3. The heme of hemoglobin is not irreversibly changed by the formation of sulfhemoglobin.

4. The solubility, resistance to alkali, molecular weight, and cataphoretic mobility of hemoglobin and its corresponding sulfhemoglobin are the same.

5. Sulfhemoglobin forms a compound with CO.

6. 1 atom of sulfur for each atom of hemoglobin iron is necessary in the conversion of hemoglobin to sulfhemoglobin. Sulfhemoglobin contains 1 atom of labile sulfur, which is not in the form of a free sulfhydryl group.

7. Reduced sulfhemoglobin is very stable, but the oxidized form is unstable.

8. A sulfhemoglobin can be formed from muscle hemoglobin. Its stability, properties, and CO compound are described.

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A CRITICAL STUDY OF THE THERMOELECTRIC METHOD OF MEASURING VAPOR PRESSURE*

By RAYMOND R. ROEPKE† AND EDWARD J. BALDES

(From the Division of Physics and Biophysical Research, The Mayo Foundation, Rochester, Minnesota)

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The thermoelectric method, as devised by Hill (1), is a dynamic method of comparing the vapor pressure or the activity of the water of two solutions. The apparatus consists essentially of a symmetrically wound thermopile of 50 to 70 junctions which is so designed that two filter papers, one moistened with the sample and the other with a standard or reference salt solution, may be placed on opposite sides of the thermopile in contact with the two rows of thermojunctions. A difference in the rate of evaporation from (or condensation on) the two solutions results in a difference in temperature of the two rows of junctions and hence a deflection of the galvanometer to which the thermopile is connected.

In Baldes' modification of the thermoelectric method ((2, 3) and unpublished data), a sensitive thermocouple replaces the thermopile and, because of the simplicity of construction and greater efficiency of the thermocouple, this paper will deal chiefly with the modified method. This method has been used to determine the vapor pressure of biologic fluids (4, 5) and the molecular weight of inulin (6). The apparatus consists of two thermocouples (permitting duplicate determinations) placed at right angles to each other, the thermojunctions being made in the form of loops and insulated with a bakelite lacquer. The thermo-

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† Fellow in Biophysics.

couples, which are connected to a sensitive galvanometer through double pole, double throw switches, are enclosed in an air-tight chamber and placed in a water bath. The method consists in measuring the deflection of the galvanometer obtained by placing a drop of the sample, for example, blood, on one junction of a thermocouple and a drop of a reference salt solution on the opposite junction, the inner surface of the chamber being lined with filter paper moistened with the reference solution. The deflection of the galvanometer is taken as a measure of the difference in vapor pressure between the blood and the reference solution and is calibrated by placing solutions of known concentrations on opposite junctions of the thermocouple. In order to eliminate errors resulting from asymmetry of the moist chamber and from parasitic E.M.F.'s in the circuit, the measurement is repeated with solutions reversed on the thermocouple, the average of the two deflections being taken as a measure of the difference in vapor pressure of the two solutions. Since it is most convenient to express the results in terms of the concentration of the reference solute and since the vapor pressure as well as the activity of the water in the solution varies inversely as the concentration of solute, it is sometimes convenient to use the term "osmotic pressure." It is to be understood, however, that by osmotic pressure is meant a measure of the activity of the water in the solution and that two solutions in which the water has the same activity and hence the same vapor pressure are considered as having the same osmotic pressure. Thus a solution with an osmotic pressure of 10 mm NaCl is one in which the activity of the water or the vapor pressure is equivalent to that of a solution containing 10 mm of NaCl per kilo of water.

Culbert, McCune, and Weech (7) attempted to show that the thermoelectric method gave correct values for the vapor pressure of serum by making measurements of the vapor pressure (with the thermopile) on 53 samples of human serum and comparing with the equivalent vapor pressure as calculated from the concentrations of total base, non-protein nitrogen, and protein. By statistical treatment of their results, they were able to conclude that the rate of evaporation of serum was directly proportional to the difference between the vapor pressure of the serum and that of the solution on the walls of the chamber. They could

not show, however, that the constant of proportionality was the same for serum as for the reference solution. That is, their procedure does not eliminate the possibility of a constant error due, for example, to surface films.

In considering the errors inherent in the thermoelectric method we might list the following possibilities: (1) surface films, (2) difference in coefficient of diffusion of water in the sample from that in the reference solution, (3) greater non-solvent volume in the sample than in the reference solution, (4) difference in the shape of drops, owing to difference in surface tension, (5) difference in heat of condensation, (6) thermochemical reactions in the sample, and (7) the presence of volatile solutes.

In determining the vapor pressure or osmotic pressure of biologic fluids, the apparatus is usually calibrated by placing a drop of 171.1 mM NaCl (1.0 gm. of NaCl per 100 gm. of H_2O) on one junction of a thermocouple with 154.0 mM NaCl (0.9 gm. of NaCl per 100 gm. of H_2O) on the opposite junction and on the walls of the chamber. At the steady state, water will be condensing on the drop of 171.1 mM NaCl at a rate such that the heat of condensation is equal to the heat lost from the drop by conduction, radiation, and convection. The water which condenses on the drop passes through the surface, diluting the surface layer and thence diffusing inward to dilute the entire drop. Thus there will be a concentration gradient in the drop which will depend on the rate of condensation and on the coefficient of diffusion of water in the solution. If the drop of 171.1 mM NaCl is replaced with a drop of blood, which is isosmotic with 171.1 mM NaCl, one may expect the deflection of the galvanometer to be appreciably less if surface films of proteins or lipids interfere with the passage of water through the surface or if the coefficient of diffusion of water in blood is less than that in the solution of NaCl. Also, since the drop of the solution having the higher osmotic pressure is being constantly diluted, a drop of blood may be expected to show a greater decrease in osmotic pressure than that of the reference solution, since the blood contains a greater amount of non-solvent material. It is assumed, of course, that the time allowed for equilibration of the sample is the same as that allowed for the reference solutions used in calibrating the galvanometer deflection.

A drop of salt solution is held on the loops of the thermocouple in a nearly spherical form while a drop of blood or cells, owing to its lower surface tension and greater density, tends to flatten out and may completely surround the thermojunction. If the lower portion of the junction remains uncovered, as appears to be the case with drops of salt solution, the junction may not be maintained at the same temperature as that of the drops, owing to loss of heat from the uncovered portion of the junction. Thus, if a drop of the sample entirely surrounds the junction, the deflection may be greater than that obtained with the isosmotic reference solution. If the thermocouples are used with blood or serum for a period of a week or more, the units become coated with protein or lipid material and the drops spread or "creep" a considerable distance along the stem. In order to prevent this it has been found desirable to cover the units with a thin coat of paraffin by applying a saturated solution of paraffin in ether with a small brush. However, this does not eliminate the possibility of error due to difference in surface tension.

Although it need not be considered in the usual determination of the vapor pressure of dilute solutions, a difference between the heat of condensation of the sample and the reference solution may warrant consideration in some applications of the thermoelectric method, perhaps in a study of concentrated solutions, in which case the heat of dilution may amount to an appreciable fraction of the heat of condensation. Other conditions being equal, the greater the heat of condensation, the greater will be the deflection of the galvanometer.

These five possible sources of error are those due to the dynamic nature of the method. Thus we can evaluate the error due to the combination of these factors by comparing the osmotic pressure of the sample, for example, blood, against that of the reference solution, first under nearly static conditions and then under dynamic conditions. In the usual procedure of determining osmotic pressure, the blood is placed on one loop of each pair of thermocouples and a reference solution on the opposite loops and on the walls of the chamber. That is, the solution on the walls is always isosmotic with the reference solution with which the sample is being compared. Thus if the osmotic pressure of the blood differs by 17.1 mm NaCl from that of the reference solution,

the blood on one loop of a thermocouple will differ in osmotic pressure by 17.1 mm NaCl from the solution on the walls of the chamber and on two loops of the same thermocouple unit; that is, the drop of reference solution on the opposite loop of the same couple and on one loop of the adjacent couple. In determining the error due to surface films, and so forth, this condition is approximated very closely by placing on the loops of one thermocouple drops of the same solution that is on the walls of the chamber, on one loop of the adjacent thermocouple, a drop of the blood, and on the opposite loop, a reference solution that is isosmotic with the blood. If under these conditions the deflection of the galvanometer is zero when the solution on the walls is the same as that on the loop opposite the drop of blood, water is neither evaporating from nor condensing on the blood (assuming that there are no volatile solutes or thermochemical reactions occurring in the blood); hence there could be no error due to any one of the five factors which have been mentioned. If the solution on the walls of the chamber (and on the two adjacent loops) then is replaced with water, water will evaporate from the walls and from the adjacent loops and condense on the blood and the isosmotic salt solution. If, as a result of the factors that have been listed, the temperature of the junction supporting the blood is not increased (above the temperature of the bath) to the same extent as that of the opposite junction, the galvanometer will be deflected in the direction which indicates that the blood has an osmotic pressure less than that of the isosmotic salt solution. If, on the other hand, the solution on the walls and the adjacent loops is replaced with a hypertonic solution, water will evaporate from the blood and the isosmotic salt solution and, as a result of the same factors, the temperature of the junction supporting the blood will not be decreased to the same extent as that of the opposite junction, the blood appearing to have an osmotic pressure higher than that of the salt solution. In this manner one can determine the error due to the combination of the first five factors that have been listed under various experimental conditions.

Since the thermocouple cannot distinguish between heat of reaction and heat of condensation, the occurrence of exothermic reactions in the sample will result in too high a value for the osmotic pressure. Endothermic reactions would result in an error

in the opposite direction. Meyerhof (8) has shown by means of a calorimetric method that the production of heat in mammalian blood occurs chiefly if not entirely within the cells. Thus it should be possible to obtain some idea of the error due to heat production in blood cells by comparing the osmotic pressure of the serum against that of the intact, centrifuged cells. This was done by Bateman (9) with a thermopile, with human blood equilibrated with 5 per cent CO_2 . The average of five determinations indicated that the osmotic pressure of the cells was less than that of the serum by 0.8 mm NaCl, a difference which is opposite from that which should be obtained if the heat production in the cells were greater than that in the serum. It has been deemed advisable to repeat Bateman's work by using the modified method and blood of various species.

If the sample contains a volatile solute, its apparent vapor pressure will be too high unless the solute is in equilibrium with its vapor in the chamber. In normal blood, the only volatile solutes present to any extent are the gases. Margaria (10) has shown that the osmotic pressure of whole blood is from 4 to 6 per cent less in air than in 5 per cent CO_2 (the approximate CO_2 tension of arterial blood). However, the deflection of the galvanometer is constant within 30 minutes (except for slight variations due, most likely, to temperature changes in the bath); hence it is only necessary to make corrections for the amount of CO_2 dissolved in the reference solution. This was calculated by Culbert (11) to amount to an equivalent of 0.6 mm NaCl in a chamber containing 5 per cent CO_2 . The effect of volatile solutes will not be considered further although it must be kept in mind when considering various applications of the thermoelectric method.

EXPERIMENTAL

The error involved in determining the osmotic pressure of blood cells under dynamic conditions was determined according to the procedure that has been outlined. Since the percentage of error appears to be the same whether the solution on the walls is hypotonic or hypertonic, it can be evaluated by determining the deflection of the galvanometer obtained by comparing the cells against a nearly isosmotic NaCl solution (usually 154 mm NaCl)

on one thermocouple of a double unit, first with water on the walls (and on the adjacent loops of the same unit), then with 308 mM NaCl on the walls, and, in order to correct for changes in osmotic pressure of the cells with time, the deflection is again determined with water on the walls. The average difference between the deflections obtained with water and 308 mM NaCl on the walls is multiplied by the calibration factor and by 100 and divided by 308 in order to express the error as per cent of the difference in osmotic pressure between the cells and the solution on the walls of the chamber. This procedure was followed in obtaining the values given in Table I, except those given for Samples 31 and 43. The value given for Sample 31 and the first series of values given for Sample 43 were obtained by comparing the deflections given with 154 mM NaCl and then with water on the walls and adjacent loops; and the second series of values, by placing 308 mM NaCl and then 154 mM NaCl on the walls and adjacent loops (corrections being made for change in osmotic pressure of the cells with time). The cells were obtained by centrifugation at about 3000 R.P.M. for 20 to 40 minutes except for Sample 99 which was centrifuged for 80 minutes. Hemolysis of the cells was obtained by freezing and thawing. The results obtained with a sample of egg yolk are also included in Table I. Positive values signify that the difference in osmotic pressure is greater than that indicated by the deflection of the galvanometer, that is that the deflection is too low; negative values indicate the reverse.

The error introduced by heat production within the blood cell was evaluated by comparing the osmotic pressure of the serum against that of the intact, centrifuged cells. Since it is difficult to maintain a definite CO₂ tension during separation of the cells, it was deemed advisable to equilibrate the blood with air before centrifugation and make the comparison in a chamber containing air. The results obtained with blood of various species are shown in Table II.

Comment

As indicated in Table I, the error due to surface films, difference in coefficient of diffusion of water, and so forth, would be a negligible fraction of the total osmotic pressure of even such

viscous mixtures as centrifuged blood cells or egg yolk if the determination were made by comparing the sample against an approximately isosmotic reference solution. If the osmotic pressure of a sample of blood cells differed from that of the refer-

TABLE I
*Error in Determination of Difference in Osmotic Pressure between Blood Cells and a Reference NaCl Solution**

Sample No.	Kind of blood cells	Percentage error				Average
		A†	B†	C†	D†	
31	Horse, intact				1.5	1.5
43	Dog, intact	1.7	2.1	2.0	1.2	1.7 _s
	“ “	1.9	1.6	1.7	1.7	1.7 ₂
99	“ “	2.1	2.1	1.3	2.3	1.9 _s
	“ hemolyzed‡	-0.1	-0.7	-0.4	-0.7	-0.5
98	Human, hemolyzed	0.4	1.2	1.1	1.1	0.9 _s
77§	Sheep, intact				1.9	1.9
93	“ “	1.1			0.6	0.8 _s
	“ hemolyzed	1.1	1.0	0.1		0.7 _s
93§	“ intact		1.0		2.2	1.6
	“ hemolyzed	1.9		1.3		1.6
100	“ intact	1.0	1.1	1.0	1.6	1.1 ₇
	“ hemolyzed	0.6	0.3	0.3	0.7	0.5
	Egg yolk	0.5	0.1	0.7	1.0	0.6
Average, intact cells.....						1.5 _s
Hemolyzed cells.....						0.9 _s

* The solution on the walls of the chamber was isosmotic with the reference solution.

† A, B, and C refer to the values obtained with the units in the large moist chamber, while D refers to those obtained with the small, individual chamber type of unit.

‡ A considerable amount of hemoglobin crystallized following hemolysis of cells; not included in the average.

§ Cells washed three times with 9.5 per cent sucrose.

ence solution by 10 per cent, the osmotic pressure as determined with the thermocouple would be in error by less than 0.2 per cent (assuming that the solution on the walls is isosmotic with the reference solution). The error might be of significance, however, if one wished to measure as accurately as possible the difference

in osmotic pressure between a sample of cells and the same sample to which had been added a definite amount of solute. In the case of intact blood cells, the deflection of the galvanometer would be too low by 1.5₃ per cent (average of the values given in Table I) and the corrected deflection could be obtained by dividing the measured deflection by 0.984₇ or multiplying by 1.015₃. In the case of whole blood or serum the error is too small to be determined accurately but amounts to less than 0.4 per cent and prob-

TABLE II

Apparent Difference in Osmotic Pressure between Serum and Centrifuged Cells of Blood Equilibrated with Air

Sample No.	Kind of blood	Apparent Δ osmotic pressure*
		<i>mM NaCl per kg. H₂O</i>
36	Dog	0.7
71	"	1.2
41	Human	1.7
72	"	0.7
	"	1.0†
81	"	1.2
	"	1.5†
42	Horse	0.7
44	"	0.2
45	Rabbit	1.9
46	"	3.6
50	"	1.4
53	Chicken	1.2
Average.....		1.3

* Osmotic pressure of intact cells minus osmotic pressure of serum.

† Cells washed with 0.9 per cent NaCl and supernatant wash fluid compared against washed cells.

ably to less than 0.2 per cent. With intact, centrifuged cells the error appears to be slightly greater than with the same cells hemolyzed, although it is not unduly large even with closely packed cells such as Sample 99, in which case the centrifuged cells were transparent. There appeared to be no measurable difference between the results obtained with the paraffined thermocouple units and those obtained with the untreated units.

As shown by the results given in Table II, the osmotic pressure

of intact, centrifuged blood cells appears to be consistently greater than that of the corresponding serum, owing, apparently, to heat production within the cells. While the error is relatively small in the case of blood cells, with the exception perhaps of rabbit cells, it is appreciable in the case of tissue cells which have a high metabolic rate. Although there does not appear to be a practical way to eliminate the error due to exothermic reactions or to determine exactly the amount of error in all cases, the error may be reduced appreciably by making the determination at a lower temperature (reducing the rate of heat production) and under reduced pressure (reducing the error due to a definite rate of heat production by increasing the rate of diffusion of water between the solutions in the chamber). Although such a procedure would not permit accurate determination of the osmotic pressure of tissue cells, it might be possible to measure fairly accurately the difference in osmotic pressure between two samples of tissue which have approximately the same rate of heat production. The value for the osmotic pressure of ground muscle tissue as determined with the thermocouple at 0° corresponds to 5.0 to 9.0 mM NaCl less than that obtained when the determination is made at 25° and is further reduced an equivalent of 2.5 to 5.5 mM NaCl when the chamber is partially evacuated at 0° . In making the determination at 0° , the samples were transferred to the loops in a refrigerated room ($0-4^{\circ}$) and the chamber placed in a Dewar flask containing finely crushed ice and water.

Although the thermoelectric method appears to be extremely accurate in measuring differences in vapor pressure between two solutions, the accuracy which one actually obtains depends considerably upon the technique. Variations in the deflection of the galvanometer may correspond to at least ± 0.7 mM NaCl even when the determination is made under nearly static conditions. However, by observing certain precautions it is possible to reduce the variation to within ± 0.4 or ± 0.3 mM NaCl. Of particular importance is the use of an inner bath (3, 12) to reduce variations in temperature of the moist chamber and the use of a humidified box in which to transfer the drops to the loops ((3) and unpublished data of Baldes). It also is advisable to maintain the room at a temperature near that of the water bath and to eliminate

as many connections in the circuit as possible in order to reduce variations in the deflection due to changes in the parasitic E.M.F.'s.

Normally there would be no appreciable error due to evaporation while the drops are being transferred, since the order in which the solutions are transferred to the loops is reversed each time so that the percentage of change in osmotic pressure would be equal for both solutions (assuming drops of equal size) if the humidity and the time necessary to transfer the drops are always the same. This would not be true, however, if the sample contained a large amount of non-solvent material such as in the case of blood cells or egg yolk. Thus it is of particular importance that such solutions be transferred to the loops in a saturated atmosphere. It is also important that the two solutions being compared be at the same temperature when placed on the loops, although it is not of such importance that they be exactly at the temperature of the water bath.

As suggested by Hill (1) and Baldes (unpublished data), the thermoelectric method also may be used with organic solutions, since the bakelite lacquer used for insulation appears to be resistant to most organic solvents. The occasion arose recently to determine the molecular weight of an organic compound dissolved in anhydrous ethyl alcohol and the method was found to work satisfactorily. The chief difficulty encountered was that of preventing the drops from spreading down the stem because of the low surface tension of alcohol. This difficulty might be eliminated by modifying the design of the thermocouple units.

SUMMARY

The accuracy of the modified thermoelectric method when used as a dynamic method for measuring vapor pressure or osmotic pressure was determined by comparing the osmotic pressure of the sample with that of a nearly isosmotic salt solution, first under nearly static conditions, then under dynamic conditions. The method was shown to be very accurate even when used with such viscous material as centrifuged blood cells or egg yolk. The chief difficulty encountered in the application of the method to biologic material is the error due to production of heat within the sample. This error has been evaluated in the case of blood cells

of various species by comparing the serum against the intact, centrifuged cells. Attention is called to certain precautions which must be observed in order to insure the greatest accuracy in the use of the thermoelectric method.

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THE EFFECT OF TRYPSIN AND PTYALIN PREPARATIONS ON THE GONADOTROPIC ACTIVITY OF PITUITARY EXTRACTS*

BY W. H. McSHAN AND ROLAND K. MEYER

(From the Department of Zoology, University of Wisconsin, Madison)

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Extracts of the anterior pituitary hormones such as the lactogenic (1) and gonadotropic (2) hormones have been reported to be inactivated by pepsin and by trypsin, and other pituitary hormonal activities are usually considered to be destroyed by these enzymes. The gonadotropic substances from urine of pregnant women (3) and from the serum of pregnant mares (4) have been reported to be inactivated by trypsin preparations. However, it seemed that if the pituitary gonadotropic substances were studied more extensively by using amylolytic enzymes as well as relatively pure trypsin preparations additional information concerning the properties of these substances might be obtained.

EXPERIMENTAL

This report deals with the effect on certain pituitary extracts of the following: (1) two commercial trypsin preparations¹ of high tryptic activity and relatively little amylolytic activity, (2) crystalline trypsin,² and (3) two different samples of saliva. The ptyalin preparations used in these experiments were made from the two samples of saliva by filtration, centrifugation, and a second filtration. The ptyalin preparations were high in amylolytic ac-

* Supported in part by a grant from the Wisconsin Alumni Research Foundation.

¹ The two trypsin preparations were obtained from Fairchild Brothers and Foster, New York, and from the Pfanstiehl Chemical Company, Waukegan, Illinois.

² We are indebted to Dr. John H. Northrop of the Rockefeller Institute for the crystalline trypsin.

tivity, as indicated by their ability to digest starch, and they contained little or no tryptic activity.

The gonadotropic extracts used were A, an aqueous supercentrifuged extract of whole sheep pituitary powder, B, a fraction of Extract A that was soluble in 50 per cent acetone at pH 6.8, and C, a follicle-stimulating extract prepared from Extract A by a modification of the Fevold (5) method. Extract A was highly active with respect to both follicular development and luteinization of rat ovaries, Extract B which was water-clear and low in solid content showed strong follicle-stimulating activity but only slight luteinizing activity, and Extract C stimulated follicles only.

The enzyme digestions of the pituitary extracts were made for 3.5 hours at 37° and at a pH of approximately 6.5. The same quantities of the extracts were treated similarly without the enzymes and used as controls. Both control and digested extracts were assayed by injecting subcutaneously a total dose of 0.5 gm. equivalent of dry pituitary powder into normal female rats 21 days old. Each rat was injected with 0.5 cc. of the proper extract on the evening of the 1st day and twice daily with this amount on each of the 4 succeeding days. The animals were killed on the morning of the 6th day and the ovaries removed, weighed, and examined grossly for the presence and relative numbers of follicles and corpora lutea. Hypophysectomized rats 21 days old were given 200 mg. equivalents of the digests daily for 10 days and were subjected to autopsy on the morning of the 11th day. The ovaries from both the normal and hypophysectomized rats were studied histologically.

DISCUSSION

The data given in Table I are representative of the results which have been obtained consistently with the different digests of Extracts A, B, and C. The administration of the commercial trypsin digests of each of the three pituitary extracts resulted in the stimulation of follicles only, as shown by observation of the ovaries at the time of autopsy. Similar results were obtained with crystalline trypsin except that the activity of Extract C was decreased to a greater extent than with the commercial trypsin. The follicle-stimulating activity of the trypsin digests of Extract

A was decreased but little when the digestion was made for 10 instead of 3.5 hours.

A luteinizing extract prepared by isoelectric precipitation and supercentrifugation was digested with commercial trypsin and

TABLE I

Data from Assays of Gonadotropic Extracts Digested with Trypsin and Ptyalin Preparations

Extract		Enzyme		No. of rats	Ovaries	
Kind	Dose	Kind	Amount		Weight, average	Response
	mg.				mg.	
A. Aqueous supercentrifuged extract of sheep pituitary powder	500			5	133	Many corpora lutea
	500	Trypsin*	20 mg.	12	71	Follicles
	500	" †	5 "	3	58	"
	500	" †	1 "	3	70	"
	500	Ptyalin	3 cc.	5	46	Many corpora lutea
	100			7	92	" " "
	100	Trypsin*	4 mg.	6	30	Follicles
	500			14	94	Few corpora lutea
B. Fraction of Extract A soluble in 50 per cent acetone at pH 6.8	500	Ptyalin	1.5 cc.	9	17	1 to 6 corpora lutea
	500	"	3 "	6	14	1 " 6 " "
	500	Trypsin*	20 mg.	6	32	Follicles
	500	" †	1 "	6	41	"
C. Follicle-stimulating extract	500			5	35	"
	500	Ptyalin	3 cc.	9	10	Negative
	500	Trypsin*	10 mg.	6	27	Follicles
	500	" *	20 "	3	24	"
Extract A + Luteinizing extract	500	" *	20 "	4	75	"
	500	" *	15 "			
Extract A + Luteinizing extract	500	" *	20 "	4	73	Numerous corpora lutea
	500	" *	20 "			

* Commercial.

† Crystalline.

injected separately but at the same time with a trypsin digest of Extract A. The ovaries stimulated by this combination contained follicles only. When the luteinizing extract was not digested with the trypsin but was injected separately and at the same time with

the trypsin digest of Extract A, the stimulated ovaries contained numerous corpora lutea. The results of this experiment which are given in Table I provide further evidence that the luteinizing activity was eliminated much more rapidly than the follicle-stimulating activity by digestion of the extracts with the trypsin preparations.

The data from preliminary experiments indicate that the gonadotropic activity of pregnant mare serum is destroyed by the action of trypsin. This confirms the results reported by Cartland and Nelson (4). We have found also that pregnant mare serum in both the untreated and purified forms is inactivated by the action of ptyalin preparations.

The gonadotropic activity of Extract A was decreased greatly on treatment with the ptyalin preparations. The ovaries stimulated by the ptyalin digests of this extract contained numerous corpora lutea. The ovaries from the rats treated with ptyalin digests of Extract B were about equal in weight to the ovaries of untreated rats. These small ovaries in most cases contained from one to six corpora lutea, as indicated by both gross and histological examination. These results make it evident that the follicle-stimulating activity of Extract B was largely destroyed by the action of the ptyalin preparations. Furthermore, the follicle-stimulating Extract C was inactivated completely by the action of the ptyalin preparations. The effectiveness of the ptyalin in the destruction of the follicle-stimulating activity evidently depends in part upon the protein content of the extract. This is shown by the incomplete inactivation of Extract A which was high in protein content as compared to the inactivation of Extracts B and C which contained relatively little protein.

Histological examination of the ovaries of the normal and hypophysectomized female rats given the trypsin-digested extracts showed that they were composed of many follicles, none of which was cystic. A few of the ovaries, however, contained small patches of lutein tissue or some hypertrophy of the theca interna. While such findings render unwarranted the conclusion that the trypsin-treated extracts are totally devoid of luteinizing activity, nevertheless treatment with the trypsin preparations under the conditions used in our experiments unquestionably eliminates to a large extent the luteinizing properties of the pituitary extracts.

A tentative explanation of the data obtained in these studies is that the luteinizing activity depends upon a protein which is readily attacked by trypsin but which is resistant to the action of ptyalin preparations. Since the follicle-stimulating activity is relatively resistant to trypsin but is readily inactivated by the action of ptyalin preparations, it is suggested as a possibility that the follicle-stimulating activity may depend upon the presence of a carbohydrate grouping. Other experiments are now in progress to test further the validity of this hypothesis.

SUMMARY

The effect of two commercial trypsin preparations, crystalline trypsin, and ptyalin preparations on the gonadotropic activity of two unfractionated extracts and a follicle-stimulating extract prepared from sheep pituitary was determined. The luteinizing activity of the extracts was largely, if not entirely, destroyed by trypsin, while it was relatively resistant to the action of the ptyalin preparations. On the other hand the follicle-stimulating activity was resistant to trypsin, while it was destroyed by the ptyalin preparations.

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METHIONINE STUDIES

I. THE REACTION OF METHIONINE AND OTHER AMINO ACIDS WITH MERCURIC CHLORIDE*

BY GERRIT TOENNIES AND JOSEPH J. KOLB

(From the Lankenau Hospital Research Institute, Philadelphia)

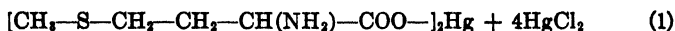
(Received for publication, August 17, 1938)

A survey (1) of the various methods that have been proposed for the isolation of natural methionine shows that all involve, at some stage, the use of mercuric chloride. It appears, however, that the nature of the reactions between methionine and mercury compounds has never been investigated. The only description of a mercury derivative of methionine was made by its discoverer (2), who reported an analysis of the precipitate formed by mercuric chloride and *l*-methionine. The result, corresponding roughly to Formula 1, methionine + Hg_2Cl_2 , was considered indicative of a "highly complex" composition. Other information bearing of the mercuric chloride precipitation of methionine is that the chloride ion has an "inhibiting effect" (3), that "the presence of sodium chloride favors the complete precipitation of the methionine" by mercuric acetate, that the precipitate is soluble in a considerable excess of mercuric acetate, that also, "if the concentration of the sodium chloride exceeds a certain value, re-dissolution of the mercury-methionine precipitate occurs" (4), and that the "precipitation of methionine as its mercury compound was unsatisfactory in the presence of large amounts of other amino acids" (5).

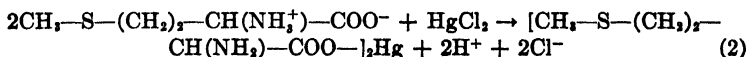
The present work was prompted by the belief that for an efficient isolation of methionine comprehension of the underlying chemistry is essential. Consideration of the published observations suggested the following working hypothesis. A compound

* Aided by a grant from the Blanche and Frank Wolf Foundation, Inc.

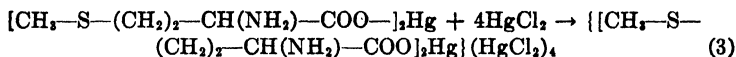
of the composition



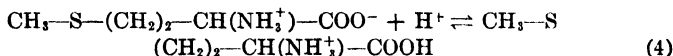
would be in accord with Mueller's (2) analysis. Its formation would depend on the availability of both mercuric ions and mercuric chloride molecules. This implies, if mercuric chloride is the sole mercury reagent used, the reactions



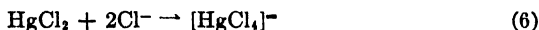
and



which involve the liberation of 1 equivalent of acid for each molecule of methionine. This acid liberation would explain why further mercuric chloride precipitation was found "very wasteful" when a purity (of isolated methionine) of 85 to 95 per cent had been attained (6), since to the extent that the system becomes less buffered by the presence of other amino acids the equilibrium



would increasingly be shifted to the right by the hydrogen ion liberated according to Equation 2. Equation 3 explains why mercuric acetate alone forms no precipitate with methionine in the absence of chloride (3) and why sodium chloride first increases and, on further addition, decreases the precipitate formation (4):



The experiments to be described confirm this view and furthermore disclose the fact that all amino acids, even those that do not form precipitates, react with mercuric chloride with liberation of acid.

Analysis of Mercuric Chloride Precipitate of l-Methionine—In an isolation of methionine, based on the method of Hill and Robson (4), a mercuric chloride precipitate was obtained which after removal of Hg and HCl gave pure (S = 21.48, 21.54 per cent) methionine in 90 per cent of the yield calculated on the basis of

Formula 1. In this mercury compound mercuric chloride was determined by titration of the acid liberated when the compound is dissolved in neutralized (methyl red) cyanide solution (7), total mercury as HgS , and total sulfur by a combined oxygen bomb-benzidine method.¹ The results, compared with those of Mueller (2) and with the values calculated according to Formula 1, are as follows, expressed in milli-units per 100 mg.:

	Hg	HgCl_2	Cl	S
Found.....	0.306	0.251		0.130
Mueller.....	0.309		0.527	0.130
Calculated.....	0.316	0.252	0.505	0.126

Mechanism of Reaction between Methionine and Mercuric Chloride—For the further experiments the *dl* form of methionine (Eastman Kodak Company) was used, unless otherwise stated.

Titration experiments, described in Fig. 1, show that in harmony with the postulate of Equation 2 the maximum amount of acid liberated corresponds to 1 equivalent per molecule of methionine. Fig. 1 further shows data on the relation between the amount of mercuric chloride used and the extent of methionine precipitation. These determinations are in agreement with the postulated composition inasmuch as the HgCl_2 indicated by the cyanide reaction (*cf.* preceding section) approaches the expected value. Evidence that this value represents the actual maximum is given in Table I. It appears then that the precipitate formation involves the two reactions described by Equations 2 and 3, and that the second of these requires a considerably larger HgCl_2 excess than the first one.

Effect of Chloride Ion—Fig. 2 shows how the acid liberated by the interaction between methionine and mercuric chloride is decreased by the presence of sodium chloride, as is to be expected from the reaction indicated by Equation 6 if HgCl_2 is a reactant according to Equations 2 and 3. A quantitative analysis of this effect, involving determination of the equilibrium constants corresponding to Equations 2, 3, and 6, could not be attempted.

Effect of Mercuric Acetate, Alcohol, and Acidity—To the extent that chloride is liberated in accordance with Equation 2 it will, according to Equation 6, decrease the effective HgCl_2 concentra-

¹ Toennies, G., and Callan, T. P., unpublished work.

tion involved in the precipitate formation, as shown by Equation 3. Consequently, it should be possible to reduce the amount of mercuric chloride required for complete precipitation (*cf.* Fig. 1) by introducing an amount of mercuric acetate equiv-

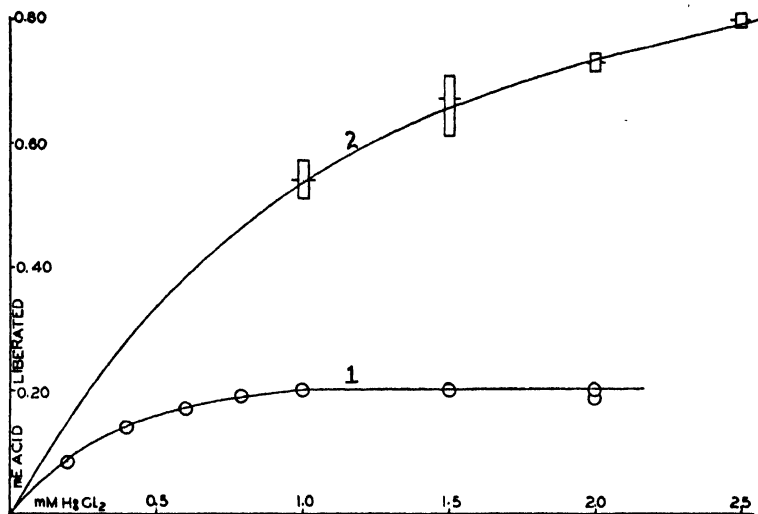


FIG. 1. The reaction between methionine and mercuric chloride. The mercuric chloride solution, in a volume of 10 to 14 cc., was neutralized to an intermediate shade of methyl red (this required 0.015 to 0.07 milliequivalent of NaOH); 1 cc. of 0.200 M methionine solution was added, causing the indicator to turn red, and the solution was titrated back to the intermediate color (pH \sim 5.5) with 0.07 N NaOH (Curve 1); final volume 16 ± 1 cc. The amount of precipitate formed increases with increasing amount of mercuric chloride. For its determination the precipitate was filtered, after standing at room temperature for 2 hours or longer, washed repeatedly with acetone, and dissolved on the filter paper with about 6 cc. of neutralized (methyl red) 0.6 M KCN solution. The resulting filtrate and washings were collected in the original flask and titrated with NaOH (each symbol on Curve 2 represents the mean value with average deviation of a group of four to eight determinations).

alent to the methionine in order to reconvert the chloride ion into HgCl_2 according to Equation 5. In connection with this point consideration was also given to the possibility of decreasing the solubility of the precipitate by the use of alcohol, which,

TABLE I

Effect of Mercuric Acetate, Alcohol, and Acidity on Precipitation of Methionine by Mercuric Chloride

The amount of methionine (0.200 mm), the total volume (16 cc. unless stated otherwise), and the method of determination are in accordance with the legend of Fig. 1. The amount of mercuric acetate, where used, was 0.100 mm. In the experiments with 75 per cent alcohol, the volume was twice that of the corresponding experiments with 50 per cent alcohol. The amount of alkali used was that required to produce an intermediate shade of methyl red, from 0.21 to 0.34 mm depending on the amount of mercuric chloride used. The pH, estimated by various indicators, was between 3.8 and 4.2 when no NaOH was used, and about 5 (± 0.5) in the other cases. In Group D the mercuric chloride was used in alcoholic solution and added after the methionine, mercuric acetate, and water, but before the NaOH; otherwise the order of addition was mercuric chloride, water, NaOH (blank), methionine, mercuric acetate, NaOH, and alcohol.

Experiment No.	HgCl ₂ mm	Additional reactants	Medium	Precipitate, per cent of theoretical amount			
				A	B	C (22 cc.)	D
1	1.0	NaOH	H ₂ O	69, 71	66, 68		
1a	1.0	"	"	62,* 63*			
2	1.0	"	50% C ₂ H ₅ OH	71	66, 67		
3	1.0	"	75% "		56, † 57†		
4	1.0	(CH ₃ COO) ₂ Hg	H ₂ O	68, 69			
5	1.0	"	50% C ₂ H ₅ OH	82, 85	73, 75		
6	1.0	"	75% "		71, † 76†		
7	1.0	NaOH + (CH ₃ COO) ₂ Hg	H ₂ O	84, 84		75, 88	
8	1.0	" + "	50% C ₂ H ₅ OH			93, 94	92, 95
9	2.0	" + "	50% "				102, 99
10	3.0	" + "	50% "				101, 102
11	4.0	" + "	50% "				98, 100
12	1.0	" + "	75% "			92, † 94†	

* L-Methionine.

† Total volume 32 cc.

‡ Total volume 44 cc.

by virtue of its capacity to dissolve 5 times as much mercuric chloride as water, would be useful also for the purpose of keeping down the total operating volume. The results of the experiments concerned with these questions, including the similarity in the behavior of racemic and natural methionine, are summarized

in Table I. Several points are brought out. First, the mercuric chloride compound of *l*-methionine seems to be slightly more soluble than that of the *dl* form (Experiment 1a, Group A). Second, while replacement of sodium hydroxide by mercuric acetate does not increase the precipitation (Experiments 1, A and 4, A), combination of the two agents causes substantial improvement (Experiment 7, A). Obviously the acetic acid resulting from the combination of Equations 2 and 5 must be neutralized for optimal

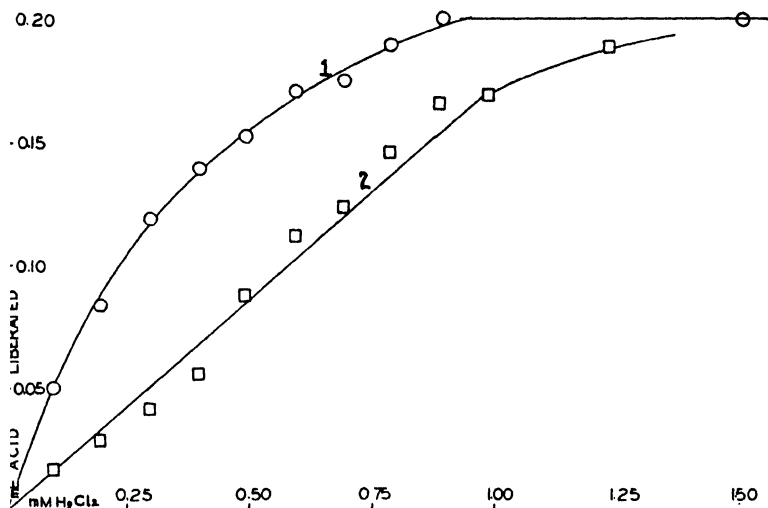


FIG. 2. The effect of chloride ion on the reaction of mercuric chloride and methionine, measured by the acid liberated. Curve 1, no NaCl added; Curve 2, 0.50 mM of NaCl. Except for the additions of NaCl, the experimental conditions were those stated in connection with Fig. 1.

precipitation (cf. Equation 4). The stated solubility of the precipitate in an excess of mercuric acetate (4) may also be attributed to the acidic reaction of the latter. Third, introduction of alcohol in the absence of mercuric acetate has no effect (Experiments 1, A, 2, A; 1, B, 2, B), except as a diluent, thereby decreasing the yield (Experiments 2, B and 3, B). However, the addition of alcohol when mercuric acetate is present appears to improve the yield (Experiments 4, A, 5, A; 7, C, 8, C), while doubling the volume with alcohol leaves the yield approximately unchanged

(Experiments 5, B, 6, B; 8, C, 12, C). In order to explain the difference in the effect of alcohol, depending upon whether or not mercuric acetate is used, the number of equilibria involved must be taken into consideration. It seems possible, for instance, that a favorable effect of alcohol on the precipitation reaction of Equation 3 may be counteracted by an unfavorable effect consisting in shifting the equilibrium of Equation 6 to the right. In the mercuric acetate experiments the latter effect would be eliminated by removal of Cl ions according to Equation 5. Fourth, confirmation of the postulated composition (Equation 3) is obtained, since the theoretical mercuric chloride content of the precipitate is not exceeded in the presence of large excess of reagent (Experiments 9, D, 10, D, 11, D).

Effect of Other Amino Acids—In connection with the isolation of methionine from hydrolysates information on the interference of other amino acids is essential. For the present purpose mercuric reactions which presumably involve specific anions other than chloride or acetate, such as sulfate, nitrate, or carbonate, or require an alkaline reaction, may be omitted from consideration, because these conditions can be avoided in the precipitation of methionine. Reactions with mercuric chloride under non-alkaline conditions, with or without precipitate formation, have been known to occur with cysteine (8) and cystine (9); and with mercuric acetate the acid amino acids aspartic acid (10) and glutamic acid (11, 12) as well as cystine (13) have been reported to form insoluble precipitates—probably simple mercuric salts of the formula $R(NH_2)(COO)_2Hg$ (11). However, that the reactive possibilities are not limited to these cases is shown by results summarized in Table II, which demonstrates the effect of the presence of various amino acids on the interaction of methionine and mercuric chloride. The figures on the acid liberation are of limited accuracy, owing to lack of sharpness in the end-point and, in some cases, anomalous indicator colors. The results, however, show beyond doubt that amino acids of different types diminish the amount of mercuric chloride available for the precipitation of methionine by engaging it in a reaction which is revealed by the liberation of acid. It should be noted that, according to Fig. 2, the amount of acid liberated by methionine alone under similar conditions is about 0.16 milliequivalent.

TABLE II

Reaction of Various Amino Acids with Mercuric Chloride

About 0.5 mm of amino acid was dissolved in a few cc. of water, and NaOH or HCl was added, when necessary, to bring the acidity into the intermediate range of methyl red. 0.50 mm of HgCl_2 (2 cc. of 0.25 M) was added and the solution was titrated with 0.07 N NaOH until the intermediate color was reestablished. 1 cc. of 0.200 M methionine was added and the solution was titrated to the same color for the third time. The total volume was 10 to 20 cc. Small amounts of methionine precipitate appeared, seemingly in proportion to the amount of acid liberated by the methionine.

Amino acid	Amount	Effect of adding HgCl_2		Acid liberated by methionine
		Acid titrated	Precipitate formed	
		m. eq.		m. eq.
<i>d</i> -Alanine.....	0.555	0.119	No	0.083
<i>d</i> -Arginine.....	0.457	0.111	Yes	0.059
<i>l</i> -Aspartic acid.....	0.512	0.126	No	0.053
" ".....	0.707	0.227		0.037
<i>d</i> -Glutamic acid.....	0.475	0.145		0.080
" ".....	0.569	0.126		0.041
Glycine.....	0.448	0.170		0.031
<i>l</i> -Histidine*.....	0.440	0.672	Yes	0.00
".....	0.564	0.673	"	0.00
<i>d</i> -Isoleucine.....	0.473	0.175	No†	0.109
<i>l</i> -Leucine.....	0.466	0.120	"	0.081
<i>l</i> -Phenylalanine.....	0.532	0.216‡	"	0.102
<i>dl</i> -Phenylalanine.....	0.460	0.268‡	"†	
<i>l</i> -Proline.....	0.584	0.114		0.078
<i>l</i> -Serine.....	0.483	0.185		0.048
<i>dl</i> -Serine.....	0.612	0.228		0.058
<i>l</i> -Tryptophane.....	0.305	0.646‡	Yes§	
<i>d</i> -Valine.....	0.558	0.125	No	0.083
<i>dl</i> -Valine.....	0.531	0.102		0.091

* The monohydrochloride was used.

† A milkiness or turbidity was caused by the addition of mercuric chloride and neutralization.

‡ Anomalous color of methyl red.

§ Very slowly.

The question arises as to the nature of this apparently general reaction of amino acids with mercuric chloride. While a complete investigation of the affinities disclosed could not be indulged in for the present, a few experiments in this direction were undertaken

which served to confirm the original observations. One series of experiments was designed to show the maximum amount of acid liberated from different amino acids by an excess of mercuric chloride, while another set aimed at the limiting value under reversed conditions; *i.e.*, the acid liberated by a constant amount of mercuric chloride in the presence of increasing amounts of amino acids. In both cases definiteness of the results was impeded by the strong buffering action in the range of the titration end-point which is produced by large amounts of either mercuric chloride or amino acids. Nevertheless certain relations are discernible in these results, the graphs of which are shown in Fig. 3. In all cases investigated of neutral, acid, and basic amino acids, except histidine, generation of 1 equivalent of acid per molecule of amino acid seems to be approached with increasing amounts of mercuric chloride. In an examination of the plotted curves the titration difficulties and the fact that no corrections have been applied for the acidity of the mercuric chloride must be considered. The latter correction is of the order of 0.0275 milliequivalent per 4 cc. of the solution used, but with the larger amounts an exact value cannot be obtained. At present no interpretation can be given to the characteristic S-shaped curves which are shown by the two simple diamino acids lysine and ornithine and are also in evidence in the case of arginine. Histidine differs sharply by its steep reaction curve which apparently exceeds the value of 2 equivalents of acid per molecule of amino acid. All four of the basic amino acids studied, but none of the neutral or acid ones, formed precipitates under the conditions of the experiment. Precipitates of amino acids of the basic group, formed by mercuric chloride under alkaline conditions, have been investigated by Vickery and Gordon (14) who found complicated relations. Analysis of the neutral precipitates so obtained would be interesting in this connection, but had to be deferred. In the case of the acid amino acids the fact that glutamic and aspartic acids form insoluble mercury salts of the type $R(NH_2)(COO)_2Hg$ (11) with mercuric acetate, while they do not yield precipitates under the present conditions, might suggest the formula $R(NH_2)COOHgCl$ for the soluble reaction products of $HgCl_2$. Verification of this supposition by determining the limiting amount of acid produced by 1 molecule of mercuric chloride in the presence of an excess of

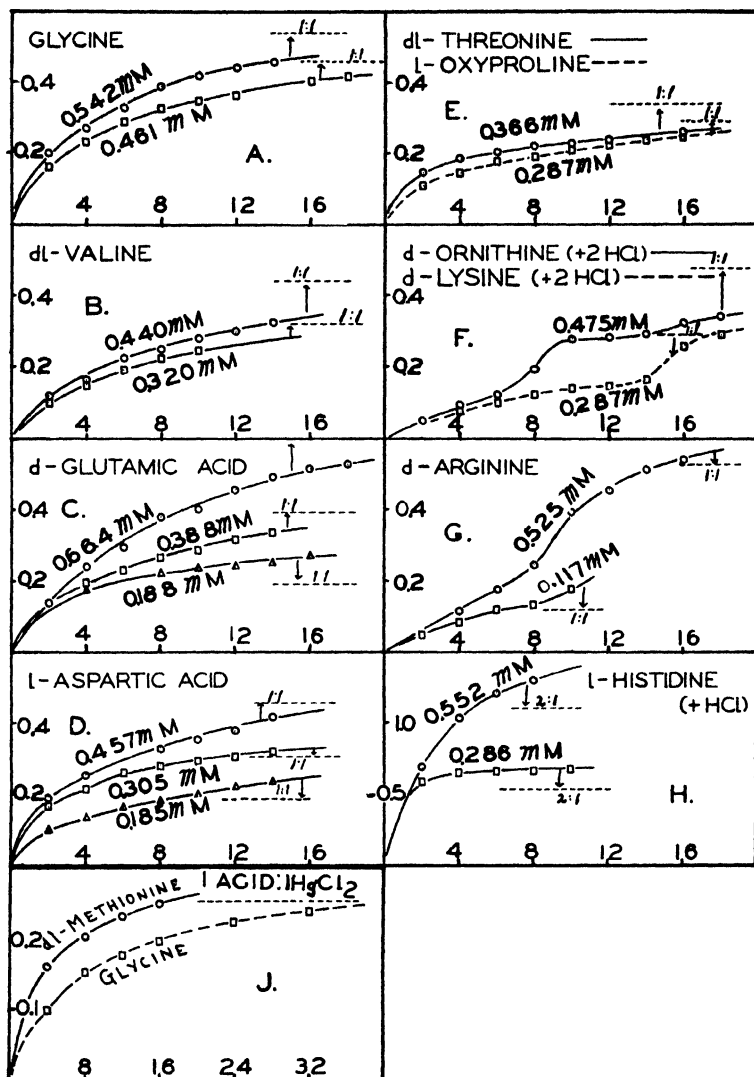
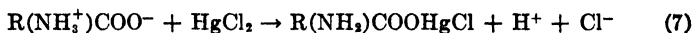
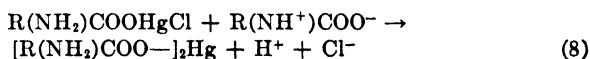


FIG. 3. Reactions of amino acids with mercuric chloride. A to H, the acidity produced by addition of successive amounts of HgCl_2 to the concentrated solution of the amino acid (neutralized by NaOH or HCl; methyl red) was titrated. I, titration of the acidity produced by addition of successive amounts of amino acid to 1 cc. of 0.25 M HgCl_2 (neutralized). The ordinate represents milliequivalents of NaOH (0.07 N) consumed, and the abscissa cc. of 0.25 M HgCl_2 , except in I where it represents cc. of 0.125 M amino acid solution.

neutralized glutamic acid proved impossible on account of the buffering action of the glutamate ion. However, data could be obtained by this type of experiment in the case of neutral amino acids. The results (Fig. 3, *J*) harmonize with the idea that at least the first step of the interaction between amino acids and HgCl_2 takes place according to the equation



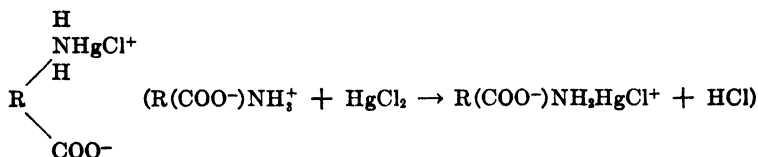
Obviously in the case of methionine this step must be followed and partly overlapped by



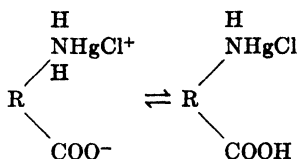
while in the case of the acid amino acids the non-appearance of a precipitate would indicate that this second step does not take place.

DISCUSSION

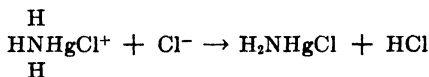
A general complex formation of mercuric mercury with amino acids appears not to have been noted previously, except for a statement by Malfatti (15) that amino acids are capable of dissolving freshly precipitated mercuric oxide. That statement would certainly imply the existence of non-ionized linkages between mercuric mercury and amino acids, at least as far as the neutral ones are concerned. The existence of such affinities hardly seems surprising when the general kinship of mercuric and cupric ions is considered, together with available observations on the formation of non-ionized linkages between amino acids and various inorganic cations of complex-forming tendencies (*cf.* Cu^{++} (16), Fe^{+++} (17), or Cr^{+++} (18)). Especially in the case of Hg^{++} the well known reactions between HgCl_2 and NH_3 suggest pronounced affinities between amino nitrogen and metal ion which would cause formation of compounds of the type



These compounds may be considered "inner salts" just as well as "salts with restricted ionization due to a covalent bond between Hg and amino group." While "dissociation" of this "inner" salt would remain an intramolecular event



the analogous reaction in the case of ammonia yields the "infusible precipitate"



As to the main subject of the present work, the conditions of formation and the nature of the mercuric chloride precipitate of methionine, the evidence presented indicates a mercuric chloride addition compound of the normal mercuric salt. On the basis of the evidence the forces responsible for the mercuric chloride addition cannot be localized within the methionine molecule. By analogy with similar compounds of simple aliphatic (19) and aromatic (20) sulfides it may only be surmised that the single sulfur atom is the center of attraction. Another possibility of analogy, *viz.* with the α -mono- and α,α' -dimercuric chloride compounds of the cyclic sulfide thiophene (21) which result from substitution of α -hydrogen by $-\text{HgCl}$, seems to be ruled out by the experimental data.

SUMMARY

Factors governing the conventional precipitation of methionine with mercuric chloride have been investigated. The composition of the precipitate and the mechanism of its formation have been elucidated. It has been found that complete precipitation is favored by neutrality, by the absence of chloride ion, by removal, with mercuric acetate, of free chloride ion formed in the reaction, and by the presence of alcohol. The basic amino acids, which form precipitates with mercuric chloride, and the acid ones, which form precipitates with the mercury ion of mercuric acetate, should

be absent in the precipitation of the mercury complex of methionine.

The common neutral amino acids do not interfere by precipitate formation, but they form soluble compounds with mercuric chloride in a reaction made patent by the liberation of acid. This effect can be compensated for by an adequate excess of mercuric chloride and by the addition of mercuric acetate and alkali.

In the experiments presented no consideration has been given as yet to the possibilities of the formation of "mixed" compounds of mercury with more than one species of amino acid.

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FURTHER STUDIES OF THE CALCIUM CONTENT OF THE BODY AS INFLUENCED BY THAT OF THE FOOD*

BY CAROLINE SHERMAN LANFORD AND H. C. SHERMAN
(From the Department of Chemistry, Columbia University, New York)

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In the first series of experimental studies made in this laboratory upon the calcium content of the body as influenced by that of the food (1-3), rats of identical previous nutritional history received different levels of calcium intake from the age of 28 to 30 days and were analyzed at fixed ages during or after the period of rapid growth. The work of Campbell, Bessey, and Sherman (4) dealt with the more slowly developing effects of a diet of moderately deficient calcium content. Toepfer and Sherman (5) began the study of the effects of liberal *versus* minimal adequate calcium intake upon the body calcium of the offspring of families on such diets; and this plan has been further developed in the present work.

By continuing the controlled comparative feeding into the second generation (or longer), one obtains much more conclusive evidence upon the far reaching biochemical problem of the extent to which nutrition influences the chemical composition of the body among the normal members of a species. The present investigation deals with the constructive aspect of this problem. The low calcium dietary of the experiments here reported is adequate, as shown by the fact that rat families are still thriving upon it in the forty-fourth generation in the hands of Dr. H. L. Campbell of this laboratory. We are therefore dealing, not with the correction of a deficiency condition (at least in the sense hitherto understood), but rather with the increase of the rate of calcification and the body calcium contents of the members of already normal families, through the enrichment of the calcium content of the diet.

* Part of the material in this paper was presented before the meeting of the American Society of Biological Chemists at Baltimore, April 1, 1938.

Here it is clearly shown that the chemical composition of the normal body can, as regards some of its constituents, be very significantly influenced by such nutritional differences as are encountered among staple food supplies. Also, the differences of body composition thus induced are shown to be more than temporary responses to an increase of intake; for they are greater in the second generation than the first, and, while greatest during the period of most rapid growth, they are still analytically measurable and statistically significant after the adult stabilization of the composition of the body as attained, respectively, upon "minimal adequate" and liberal levels of calcium intake.

The present findings have also the further significance of constituting what may be called the body composition aspect of a broader investigation into the effect of these higher levels of calcium intake upon nutritional well being throughout the life cycle of successive generations.

In drawing some of the averages here reported, certain earlier analyses carried out in this laboratory, especially by Dr. Edward Toepfer, have been included. The assistance of Mrs. Marian Linn Meinert and Miss Grace E. Guldin with the analyses is also gratefully acknowledged.

EXPERIMENTAL

Diet 16, the low calcium diet of these experiments (also called Diet A in some publications from this laboratory), consists of 5 parts of ground whole wheat and 1 part of dried whole milk powder, with added sodium chloride equivalent to 2 per cent of the wheat. Analyses of this diet as prepared from time to time show it to contain 0.19 to 0.20 per cent calcium and 0.40 to 0.43 per cent phosphorus, on the air-dry basis. The results of much work in this laboratory indicate that this is near the minimum level of calcium required by the rat for permanently satisfactory performance in all respects.

Diet 168 and Diet 169 are of identical composition with Diet 16 except for added calcium salts. In preparing Diet 168, calcium carbonate is added in amounts to raise the percentage of calcium in the food mixture to 0.64, or roughly 3 times that of Diet 16. This brings the level of calcium in the diet to that suggested by

McCollum and Simmonds as optimal for the rat. In preparing Diet 169, both calcium carbonate and secondary calcium phosphate are added, the final concentration of calcium being 0.80 per cent, and of phosphorus, 0.53 per cent.

Representative male and female young of mothers on each of these three diets were selected at 28 to 30 days, and either killed at once for analysis or continued on the diet of the mother until 2, 3, 6, or 12 months of age. They were then killed by chloroform, the gastrointestinal tract removed, the whole remaining carcass cremated, and the ash dissolved and analyzed for calcium by either the volumetric or the gravimetric modification of the McCrudden method (6), with the precautions suggested by previous work in this laboratory (1). For purposes of the present discussion, the findings are expressed both in terms of total weight of calcium in the body and in percentage of the net body weight.

DISCUSSION

Tables I and II show, for male and unmated female animals, respectively, (1) the net body weight (*i.e.*, body weight minus weight of the gastrointestinal contents), (2) the total body calcium, and (3) the calcium in percentage of the net body weight. The homogeneity of the data within each group is indicated by the customary coefficient of variability; and the means for the amount and percentage of calcium are modified by their respective probable errors.

The data are in a few cases averaged in alternative ways. The first average is always the mean for all of the analyzed animals of a given age, sex, and dietary. The second average when marked by the asterisk is the mean for all except those individuals which showed extreme variations, in either body weight or calcium content, from the usual range of their group; when marked with the dagger, those individuals are omitted which showed such variation from the general population performance as to be in our judgment not sufficiently representative for inclusion in a final mean intended to express numerically the results of a careful critique of the present data in the light of our experience with even larger numbers of animals of the same strain and on the same diets. The discussion which follows is based primarily on the data thus

edited, while a simple comparison of the alternative averages will show that the use of the means of all analyses made would lead to an essentially identical interpretation.

TABLE I
Calcium Content of Male Rats

Age	Diet No.	Ca in diet	No. of cases	Net weight		Calcium content			
						Total		Percentage of net weight	
				Mean	C.v.	Mean \pm p.e.	C.v.	Mean \pm p.e.	C.v.
<i>days</i>		<i>per cent</i>		<i>gm.</i>		<i>gm.</i>			
28	16	0.20	20	39.0	10.8	0.279 \pm 0.006	14.2	0.715 \pm 0.007	6.4
	168	0.64	30	40.3	7.9	0.385 \pm 0.003	7.4	0.956 \pm 0.004	3.7
	169	0.80	24	39.2	13.3	0.400 \pm 0.007	12.7	1.022 \pm 0.009	6.6
60	16	0.20	18	102.4	17.5	0.692 \pm 0.019	17.8	0.677 \pm 0.005	4.3
	168	0.64	21	116.6	11.8	1.128 \pm 0.014	9.0	0.971 \pm 0.006	4.5
			12†	107.9	9.1	1.075 \pm 0.016	7.8	0.998 \pm 0.006	3.1
	169	0.80	16	114.3	11.9	1.178 \pm 0.021	10.6	1.039 \pm 0.009	5.3
			13†	108.8	8.0	1.136 \pm 0.014	6.6	1.046 \pm 0.010	5.3
90	16	0.20	26	173.1	16.2	1.262 \pm 0.032	19.3	0.726 \pm 0.006	6.5
	168	0.64	31	172.5	16.3	1.765 \pm 0.030	14.2	1.026 \pm 0.005	4.1
			30*	174.6	14.8	1.785 \pm 0.028	12.8	1.025 \pm 0.005	4.2
	169	0.80	30	175.1	17.3	1.858 \pm 0.037	16.3	1.064 \pm 0.006	4.4
			28*	177.1	17.0	1.875 \pm 0.038	16.2	1.061 \pm 0.004	2.9
180	16	0.20	10	259.8	6.6	2.651 \pm 0.032	5.8	1.023 \pm 0.010	4.5
	168	0.64	20	282.2	11.5	3.137 \pm 0.050	10.8	1.113 \pm 0.006	3.5
			19*	278.3	10.0	3.083 \pm 0.038	8.0	1.112 \pm 0.006	3.5
365	169	0.80	20	288.0	8.6	3.308 \pm 0.040	8.2	1.150 \pm 0.006	3.6
	16	0.20	13	321.5	8.5	3.490 \pm 0.034	5.2	1.090 \pm 0.014	6.7
			11†	314.9	7.6	3.467 \pm 0.038	5.4	1.105 \pm 0.014	6.3
	168	0.64	14	320.6	9.0	3.839 \pm 0.038	5.5	1.203 \pm 0.016	7.2
			11†	332.5	5.6	3.906 \pm 0.037	4.7	1.176 \pm 0.010	4.3
	169	0.80	16	347.3	6.6	4.077 \pm 0.044	6.5	1.175 \pm 0.008	4.2
			14†	341.5	5.2	4.022 \pm 0.041	5.8	1.178 \pm 0.009	4.4

C. v. = coefficient of variability; p. e. = probable error of the mean.

* Mean of all except those showing extreme variations in body weight or calcium content.

† Individuals showing variations described in the text are omitted from the mean.

At 1, 2, and 3 months of age, rats reared at the two more liberal levels of calcium intake show a markedly more advanced degree of calcification than animals on Diet 16, whether considered in

terms of the total amount or in percentage of body calcium.¹ At 6 months and a year of age, the differences are smaller, but still significant. Thus, from families with a dietary calcium intake of 0.64 per cent, the male animals at 28 to 30 days of age contained 38 per cent, and the females 28 per cent greater *weight* of calcium than on an intake of 0.20 per cent calcium; at 60 days, the differences averaged 56 per cent for males and 74 per cent for females; at 90 days, 41 and 58 per cent; at 180 days, 16 and 24 per cent,

TABLE II
Calcium Content of Female Rats

Age	Diet No.	Ca in diet	No. of cases	Net weight		Calcium content			
				Mean	C.v.	Total		Percentage of net weight	
						Mean \pm p.e.	C.v.	Mean \pm p.e.	C.v.
days		per cent		gm.		gm.			
28	16	0.20	23	40.3	14.5	0.300 \pm 0.006	13.6	0.748 \pm 0.006	5.4
	168	0.64	17	40.5	12.2	0.384 \pm 0.006	10.0	0.949 \pm 0.007	4.2
	169	0.80	20	37.7	10.8	0.396 \pm 0.007	11.6	1.052 \pm 0.012	7.7
60	16	0.20	20	94.5	13.5	0.641 \pm 0.016	16.5	0.676 \pm 0.006	5.7
	168	0.64	11	106.3	11.1	1.113 \pm 0.024	10.8	1.049 \pm 0.006	3.0
	169	0.80	18	96.2	7.3	1.062 \pm 0.011	6.5	1.105 \pm 0.007	4.1
90	16	0.20	17	133.8	11.7	1.109 \pm 0.024	13.6	0.830 \pm 0.008	3.0
	168	0.64	9	150.0	5.6	1.748 \pm 0.018	4.7	1.163 \pm 0.008	3.2
	169	0.80	10	153.8	6.5	1.897 \pm 0.032	8.1	1.237 \pm 0.010	7.8
180	16	0.20	12	166.6	17.9	1.999 \pm 0.091	23.6	1.195 \pm 0.022	9.5
	168	0.64	8	190.0	6.8	2.472 \pm 0.030	5.2	1.305 \pm 0.020	6.4
	169	0.80	8	189.5	5.4	2.617 \pm 0.036	5.9	1.382 \pm 0.013	4.0

C. v. = coefficient of variability; p. e. = probable error of the mean.

respectively; and at 1 year, 13 per cent for males (no females having been analyzed at this age). Similarly, raising the calcium in-

¹ Even at the early age of 2 weeks, differences in the degree of calcification at the various levels of family intake of calcium are apparent, although at birth no significant difference has been established. The average percentage of body calcium found in twelve batches of newborn young of mothers on Diet 16 was 0.291 ± 0.002 ; and in twelve batches from mothers on Diet 169, 0.283 ± 0.004 . At 2 weeks of age, males on Diet 16 averaged 0.598 ± 0.007 per cent calcium, and on Diet 169, 0.700 ± 0.005 per cent; females 0.614 ± 0.006 per cent and 0.700 ± 0.004 per cent, on Diets 16 and 169, respectively.

take from 0.20 to 0.64 per cent increased the *percentage* of calcium in the body at 1 month of age by 34 per cent in males and 27 per cent in females; at 2 months, by 47 per cent and 55 per cent; at 3 months, by 41 per cent and 40 per cent; at 6 months, by 9 per cent in both males and females; and at 1 year, by 6 per cent in male animals. All of these differences, both in total amount and in percentage of body calcium, between rats on Diet 168 and rats on Diet 16, are of unquestionable significance when analyzed statistically by the generally accepted criteria.

When the effects of the two higher levels of calcium are compared, it appears that even Diet 168, which provides 0.64 per cent calcium, or at least 3 times the "minimal adequate" intake, does not permit the maximum rate of calcification, the percentage of body calcium from 1 to 6 months of age being raised by 3 to 11 per cent as a result of increasing the dietary calcium from 0.64 to 0.80 per cent. With the possible exception of the group of females 180 days old,² these increases also are statistically significant. At full maturity, the percentage of body calcium in male animals on Diets 168 and 169 is identical.

Critical examination by statistical analysis indicates, therefore, that the findings here reported may be regarded as conclusively established. If so, they must also be regarded as significantly modifying the long standing view which was formulated by Liebig in terms of a postulated constancy of chemical composition among at least the normal members of a species; and by Claude Bernard in his emphasis, which we must now recognize as an overemphasis, upon the *fixité* of the internal environment. Researches upon differences in other, including more soluble, body constituents as influenced by the composition of the food, as well as studies of calcium from different dietary starting points, are being undertaken in this laboratory.

SUMMARY

The effects of 3- and 4-fold enrichments in the calcium content of a diet, whose at least minimal adequacy is established by the

² The "critical ratio," i.e. the ratio of the actual difference to its probable error, is here only 3.2, whereas in present usage differences are considered "statistically significant" only when their critical ratio exceeds (3.5 or) 4, in which case the odds are better than (100 or) 142:1 that the difference is not merely accidental.

fact of its support of normal nutrition through forty-three generations of rats, were here studied in the offspring (*i.e.*, second and later generations) of parallel experimental animals.

The amounts and percentages of calcium in the bodies of these rats were determined at 1 month of age (end of infancy) and at other definite ages up to 1 year, which is sufficiently advanced into adult life for the data to show that the percentage of calcium in the body was here definitely stabilized or "plateaued."

Offspring of the families on the higher levels of calcium intake were found to have attained at 1 month of age a percentage of body calcium achieved on the original diet only at 5 or 6 months of age.

During the period of rapid growth and development, the degree of calcification on the liberal intakes of calcium (0.64 and 0.80 per cent of the dry food mixture) exceeded that on the lower dietary level (0.20 per cent) by one-quarter to one-half; and at full maturity there was a presumably permanent difference of between 5 and 10 per cent. Experiments still in progress indicate that this higher calcium content is advantageous throughout life.

Although the percentage of body calcium finally reached was the same for the two higher levels of intake, the rate of calcification was somewhat greater on the diet providing 0.80 per cent than on that with 0.64 per cent of calcium.

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HETEROGENEOUS EQUILIBRIUM OF PROTEIN SOLUTIONS

II. THE INTERACTION OF CALCIUM CHLORIDE AND OTHER SALTS WITH PROTEINS, AS DETERMINED BY A NEW TYPE OF CALCIUM AMALGAM ELECTRODE

By NORMAN R. JOSEPH

(From the Department of Physical Chemistry, Harvard Medical School, Boston)

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The potentiometric method of studying the interaction of proteins and neutral salts has certain important advantages over the two methods that have been principally employed, the solubility method and the study of membrane equilibrium. The solubility method permits the independent variation of the concentration of only one of the components, so that the equilibrium can be studied only at the concentration of a saturated solution of the other. In the method of membrane equilibrium, the concentration of both components can be varied independently, but the equilibrium is attained very slowly, and except in special cases, as Adair has shown (1), cannot be expressed simply in terms of the activity coefficients of the components. In principle, the potentiometric method is capable of giving directly salt activity as a function of protein concentration and salt concentration, from which data the effect of salt on protein activity and on the membrane equilibrium can be calculated.

Studies of this kind have already been reported for the interaction of amino acids and salts (9) and for the interaction of protein and salts (10) by means of metallic amalgam electrodes and cells without liquid junction. In the former study it was found that amalgams of the very reactive alkali and alkaline earth metals are not sufficiently stable when exposed to amino acids to yield reliable potentials except under special conditions. They are also unstable in the presence of ammonium salts and many substances

containing substituted ammonium groups. Attempts to measure calcium ion activity in the presence of protein by means of the flowing calcium amalgam electrode have met with similar difficulties (7). Accordingly, in studying the interaction of proteins with ions of the alkali and alkaline earth groups, it is necessary to find a means of protecting the amalgam from protein or other disturbing substances.

In the present paper such a method and its application to several protein systems will be described. In this method the amalgam is protected from protein by a membrane that is permeable only to electrolyte and solvent.

Cells are of the type $\text{HgMe} \mid \text{MeCl}_z \mid \text{AgCl} \mid \text{Ag}$, the junction between amalgam and solution being made through cellophane. The cell reaction is $\text{Me} + z\text{AgCl} = \text{MeCl}_z + z\text{Ag}$ (z is the valence of the cation Me).

Assuming the cell reaction to be isothermal and reversible, the electromotive force is given by the relation

$$E = E^0 - \frac{\nu RT}{N\mathbf{F}} \ln a \quad (1)$$

where a is the activity of the salt, E^0 is the standard potential of the cell, ν is the number of gm. ions formed by the dissociation of 1 gm. molecule of salt, N is the number of equivalents per mole of salt, and R , T , and \mathbf{F} are respectively the gas constant, the absolute temperature, and the Faraday constant.

Method

The cell employed is illustrated in Fig. 1. It is an H-shaped tube, in one arm of which a silver-silver chloride electrode is suspended, while in the other the amalgam electrode can be supported. The latter is prepared by sealing platinum wire into glass tubing, and sealing the latter into a calcium chloride drying tube, so that the tip of the platinum comes to within about 0.5 cm. from the mouth of the tube.

Approximately 5 cc. of amalgam are introduced into the tube, the mouth of which is then closed by a cellophane membrane. A square piece of cellophane is moistened and blotted, then stretched tightly over the mouth of the tube, and fixed to the tube by means of collodion. When the tube is inverted and suspended in the

electrode vessel, as illustrated in Fig. 1, the amalgam makes contact with the platinum wire and with the solution through the membrane. To renew the surface of the amalgam, it is only necessary to remove the tube, invert it so that the amalgam flows into the bulb, and shake the amalgam.

Silver-silver chloride electrodes were prepared by the method of Noyes and Ellis (17), and were frequently checked against each other.

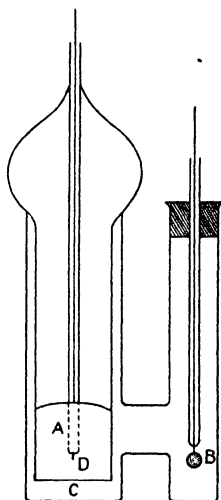


FIG. 1. Cell for determining the activity of salt in the presence of protein. *A*, amalgam electrode; *B*, silver-silver chloride electrode; *C*, cellophane membrane; *D*, platinum contact.

The amalgams were prepared by electrolysis of the corresponding chlorides, redistilled mercury being employed as cathode, in the manner described by Lucasse (13). Care was taken to minimize exposure of the amalgam to air.

In order to check the method, the activity coefficient of calcium chloride in water has been determined, and compared with the results of Lucasse (13) and Scatchard and Tefft (20), who have employed the procedure of MacInnes and Beattie (14) in which flowing amalgam makes direct contact with the solution.

Our results are given in Table I, at various values of m , the

salt molality. ($E_{0.01} - E_m$) is the difference of E.M.F. obtained when the potential given by a solution of molality m is referred to that of a 0.01 molal solution. Lucasse, in calculating the activity coefficient of calcium chloride, has taken the value 0.716 for the activity coefficient of the 0.01 molal salt, an estimate which is based on the freezing point results of Lewis and Linhart (12). Our values for the activity coefficient are based on the same reference value. As Table I shows, the results of the two methods are in good agreement to 1.0 molal calcium chloride. In pure salt solutions equilibrium across the membrane is usually established within 5 or 10 minutes after immersion of the amalgam electrode.

TABLE I
Activity Coefficient of Calcium Chloride, Obtained from Electromotive Force of Cell, $HgCa_x \mid CaCl_2 \mid AgCl \mid Ag$

m	$E_{0.01} - E$	γ	γ (Lucasse (13))*	γ (Scatchard and Tefft (20))*
<i>mole per kg. H_2O</i>	<i>volt</i>			
0.01	0.0000	(0.716)†	(0.716)†	(0.725)
0.02	0.0230	0.659	0.655	0.658
0.05	0.0450	0.567	0.569	0.570
0.10	0.0764	0.521	0.516	0.515
0.20	0.0994	0.488	0.480	0.481
0.50	0.1360	0.515	0.499	0.519
1.00	0.1770	0.706	0.709	0.715

* Observed with flowing amalgam. The results of Fosbinder (7) are in good agreement with both sets of observations up to 1.0 M $CaCl_2$.

† Reference value based on freezing point data (12).

The observed potentials often have a tendency to drift. This usually indicates a certain amount of oxidation at the surface of the amalgam. The potentials are restored to the initial value when the amalgam is shaken and a fresh surface restored. By such means it is usually possible to obtain reproducible potentials that vary only within 0.5 millivolt. Each E.M.F. that is given represents the mean of four or more successive approximately constant readings taken over a period of at least 15 minutes. Most of them were carried out in duplicate. The agreement of the results obtained by this method with those of the flowing amalgam technique indicates that there is no significant potential introduced into the cell by the membrane itself.

Another check of the method in a protein system may be obtained by studying the activity of zinc chloride in the presence of isoelectric gelatin, and comparing the results with those obtained with flowing amalgams (10). This comparison is presented in Table II. It also indicates good agreement between the two methods, and is evidence that no error is introduced into the observed *E. M. F.* by the membrane itself.

In the case of membrane equilibrium in isoelectric protein solutions it is well known that the membrane potential may amount to many millivolts (15). It is therefore important to consider the

TABLE II

Interaction of Zinc Chloride and Gelatin Determined by Two Types of Zinc Amalgam Electrodes

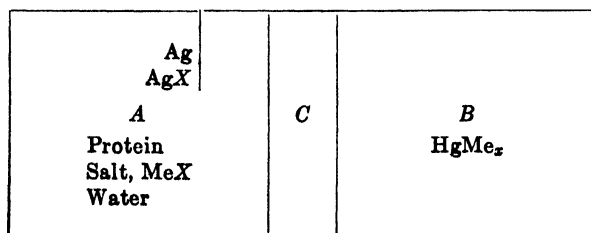
m_1	Protein	$E_p - E_0^*$	E_{\dagger}
<i>mole salt per kg. H₂O</i>	<i>gm per kg. H₂O</i>	<i>volt</i>	<i>volt</i>
0.01	50	0.0064	0.0070
0.01	75	0.0098	0.0100
0.04	50	0.0030	0.0034
0.04	75	0.0048	0.0050
0.10	50	0.0019	0.0016
0.10	75	0.0028	0.0024
0.25	50	0.0012	
0.25	75	0.0017	

* Observed with the electrode making contact through the membrane.

† Observed or estimated from data with flowing zinc amalgam electrode at 37° (10).

relation between the usual type of membrane potential and the potential measured in these experiments.

The accompanying diagram illustrates the type of equilibrium with which we are dealing.



It is a condition of equilibrium of this system that the chemical potential of the salt $\bar{F}_{\text{MeX}(A)}$ in phase A must be equal to $\bar{F}_{\text{MeX}(C)}$, the chemical potential of salt within the membrane C . Let us consider the change of state resulting from the passage of 1 equivalent through the cell, assuming it to function isothermally and reversibly. At the junction of membrane and the amalgam electrode B , 1 equivalent of the cation Me is formed. The corresponding single electrode potential is

$$E_{\text{Me}(C)} = E_{\text{Me}}^0 - \frac{RT}{NF} \ln a_{\text{Me}(C)} \quad (1-a)$$

where $a_{\text{Me}(C)}$ is the activity of cations within the membrane, and E_{Me}^0 is the standard single electrode potential of the amalgam.

At the Ag,AgX electrode, 1 equivalent of the anion X is formed and the electrode potential is

$$E_{X(A)} = E_X^0 - \frac{RT}{NF} \ln a_{X(A)} \quad (1-b)$$

where $a_{X(A)}$ is the activity of anions in the solution and E_X^0 is the standard potential of the electrode.

To fulfil the condition of electrical neutrality in phases A and C , it is necessary that x equivalents of the cation migrate from C to A , and that $(1 - x)$ equivalents of the anion migrate from A to C . The net process is the isothermal and reversible formation of x equivalents of MeX in phase A and $(1 - x)$ equivalents within the membrane. It is evident that x is a function of the relative quantities of electrolyte in A and C , and that as the ratio of the volume of A to that of C approaches infinity x approaches unity. This condition can be assumed to be met in the system we are considering, for C is a thin membrane and contains a very small mass of electrolyte in comparison with the relatively large mass in A . Thus for every equivalent passing through the cell, 1 equivalent of the cation Me migrates from C to A , while an infinitely small quantity of the anion X is transferred to C .

At the membrane there must be a potential given by the relation

$$E \text{ (membrane)} = \frac{RT}{NF} \ln \frac{a_{\text{Me}(C)}}{a_{\text{Me}(A)}} \quad (1-c)$$

This is the potential determined in studies of membrane equilibrium. Adding the three potentials given by Equations 1-a, 1-b, and 1-c, one finds

$$E = E^0 - \frac{\nu RT}{nF} \ln a_{M_0X(A)}$$

where $E^0 = E_{M_0}^0 + E_X^0$.

This is identical with Equation 1. The E.M.F. of the cell is determined therefore by the activity of salt in *A* and by the standard electrode potentials, and is not affected by the membrane itself as long as the ions within the membrane are in equilibrium with those in the solution. Under these conditions the cell reaction can be regarded simply as the formation of salt in the solution from the metal and the silver salt, the free energy change of the process being independent of the steps.

Application to Protein Solutions

Determinations of salt activity have been obtained in solutions of the following proteins: horse carboxyhemoglobin, horse serum albumin, pseudoglobulin, and gelatin. The serum albumin and pseudoglobulin were electrodialed preparations for which I am indebted to Dr. John D. Ferry. The former was Preparation IIID₂ described by Ferry and Oncley (6). An approximately 5 per cent solution had a specific conductivity of 52×10^{-6} reciprocal ohm. The dielectric increment per gm. of this fraction per liter of solution has been found to be 0.29. A 2 per cent solution of the pseudoglobulin had a specific conductivity of 9×10^{-6} reciprocal ohm. The dielectric increment per gm. of this fraction per liter of solution is 0.9,¹ at infinite dilution of protein. This is somewhat lower than the value at infinite dilution obtained for the fractions described by Ferry and Oncley, and indicates the presence in this preparation of small amounts of other globulins.

Carboxyhemoglobin was prepared from horse red blood cells according to the directions of Stadie and Sunderman (21), with a different method of electro dialysis which will be described elsewhere. The specific conductivity of an approximately 1.5 per cent solution was 45×10^{-6} reciprocal ohm.

Gelatin was prepared according to the directions of Northrop

¹ Ferry, J. D., unpublished data.

and Kunitz (16). A 5 per cent solution had a specific conductivity of 19×10^{-6} reciprocal ohm.

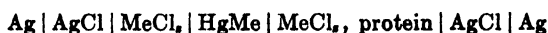
The potentiometric results for various protein-salt mixtures are given in Tables III to VI. The value ($E_p - E_0$) represents the

TABLE III

Interaction of Gelatin with Salts as Determined from Electromotive Force of Cell, $HgMe_x \mid MeCl_x \mid AgCl \mid Ag$ at 25°

Salt	m_s	m_p	$E_p - E_0$	$-\text{Log } \frac{\gamma_s}{\gamma_s^0}$	$\alpha = -\frac{\log \gamma_s/\gamma_s^0}{m_s m_p^{-1}}$
	<i>mole salt per kg. H₂O</i>	<i>mole protein per kg. H₂O</i>	<i>volt</i>		
CaCl ₂	0.01	0.00065	0.0034	0.038	5.9
	0.01	0.0013	0.0062	0.070	5.4
	0.04	0.00065	0.0016	0.018	5.5
	0.04	0.0013	0.0034	0.038	5.9
	0.10	0.00065	0.0010	0.011	5.5
	0.10	0.0013	0.0018	0.020	4.9
	0.25	0.00065	0.0006	0.007	5.2
	0.25	0.0013	0.0010	0.011	4.2
Average.....					5.3 ± 0.5
MgCl ₂	0.01	0.00081	0.0044	0.050	6.1
	0.04	0.00081	0.0020	0.022	5.6
	0.10	0.00081	0.0012	0.014	5.3
	0.25	0.00081	0.0005	0.006	(3.5)
Average.....					5.6 ± 0.3
ZnCl ₂	0.01	0.00081	0.0064	0.072	8.9
	0.01	0.00122	0.0098	0.110	9.0
	0.04	0.00081	0.0030	0.034	8.4
	0.04	0.00122	0.0048	0.054	8.9
	0.10	0.00081	0.0019	0.022	8.5
	0.10	0.00122	0.0028	0.032	8.3
	0.25	0.00081	0.0012	0.013	8.2
	0.25	0.00122	0.0017	0.019	8.0
Average.....					8.5 ± 0.3

difference in E.M.F. of a protein solution referred to an isomolal pure salt solution. It is the potential of the double cell



given by the relation

$$E_p - E_0 = -\frac{RT}{nF} \ln \frac{\gamma_s}{\gamma_s^0} \quad (2)$$

the salt molality, m_s , being the same in the protein solution and the reference solution. In Equation 2 γ_s denotes the mean ionic

TABLE IV
Interaction of Carboxyhemoglobin with Salts at 25°

Salt	m_s	m_1	$E_p - E_0$	$-\text{Log} \frac{\gamma_s}{\gamma_s^0}$	$a = -\frac{\log \gamma_s/\gamma_s^0}{m_s m_1^{-\frac{1}{2}}}$
	<i>mole salt per kg. H₂O</i>	<i>mole protein per kg. H₂O</i>	<i>volt</i>		
CaCl ₂	0.01	0.00042	0.0010	0.011	2.7
	0.04	0.00042	0.0006	0.007	3.2
	0.10	0.00042	0.0002	0.002	1.5
Average.....					2.5 ± 0.7
NaCl	0.01	0.00042	0.0006	0.005	1.2
	0.04	0.00042	0.0002	0.002	1.0
Average.....					1.1

TABLE V
Interaction of Serum Albumin with Calcium Chloride at 25°

m_s	m_1	$E_p - E_0$	$-\text{Log} \frac{\gamma_s}{\gamma_s^0}$	$a = -\frac{\log \gamma_s/\gamma_s^0}{m_s m_1^{-\frac{1}{2}}}$
<i>mole salt per kg. H₂O</i>	<i>mole protein per kg. H₂O</i>	<i>volt</i>		
0.01	0.001	0.0020	0.023	2.3
0.01	0.002	0.0034	0.038	1.9
0.04	0.001	0.0010	0.011	2.3
0.04	0.002	0.0018	0.020	2.0
0.10	0.001	0.0004	0.004	1.5
0.10	0.002	0.0008	0.009	1.5
0.25	0.002	0.0006	0.007	1.7
Average.				2.0 ± 0.4

activity coefficient of salt in the protein solution, γ_s^0 the same function in the reference solution.

It has been found empirically by earlier studies that $-\log \gamma_s/\gamma_s^0$

is approximately proportional to $m_3^{-1}m_2$. Accordingly, values of the coefficient, a , are given in Tables III to VI as the ratio

TABLE VI
Interaction of Pseudoglobulin with Salts at 25°

Salt	m_3	m_2	$E_p - E_0$	$-\log \frac{\gamma_2}{\gamma_2^0}$	$a = -\frac{\log \gamma_2/\gamma_2^0}{m_2 m_3^{-1}}$
	<i>mole salt per kg. H₂O</i>	<i>mole protein per kg. H₂O</i>	<i>volt</i>		
CaCl ₂	0.01	0.0006	0.0058	0.065	10.9
	0.01	0.0009	0.0080	0.090	10.0
	0.04	0.0006	0.0027	0.030	10.0
	0.04	0.0009	0.0036	0.041	9.1
	0.10	0.0006	0.0018	0.020	10.5
	0.10	0.0009	0.0024	0.027	9.5
	0.25	0.0006	0.0010	0.011	9.2
	0.25	0.0009	0.0014	0.016	9.0
Average.....					9.8 ± 0.5
NaCl	0.01	0.0006	0.0050	0.042	7.0
	0.04	0.0006	0.0026	0.022	7.3
	0.10	0.0006	0.0014	0.012	6.3
	0.25	0.0006	0.0008	0.007	5.8
Average.....					6.6 ± 0.6

TABLE VII
Influence of Salts on Activity Coefficients of Proteins

Values of $-\log \gamma_2/\gamma_2^0$ are computed from Equation 4 by means of experimentally determined values of a (Tables III to VI).

Ionic strength, μ ($\Gamma/2$)	Gelatin			Carboxyhemoglobin		Serum albumin, CaCl ₂	Pseudoglobulin	
	CaCl ₂	MgCl ₂	ZnCl ₂	CaCl ₂	NaCl		CaCl ₂	NaCl
0.03	3.2	3.4	5.1	1.5	0.7	1.2	5.9	4.6
0.12	6.4	6.7	10.2	3.0	1.4	2.4	11.8	9.2
0.30	10.0	10.6	16.1	4.7	2.2	3.8	18.6	14.5
0.75	15.9	16.8	25.5	7.5		6.0	29.4	

$-(\log \gamma_2/\gamma_2^0)/(m_3^{-1}m_2)$. It is approximately constant for all the systems studied. Hence, as a first approximation

$$-\log \gamma_2/\gamma_2^0 = a m_3^{-1} m_2 \quad (3)$$

where a is characteristic of the system. It has been shown earlier

(10) that Equation 3 is correlated by thermodynamics with the equation

$$-\log \gamma_2/\gamma_2^0 = 2\nu am_2^{\frac{1}{2}} \quad (4)$$

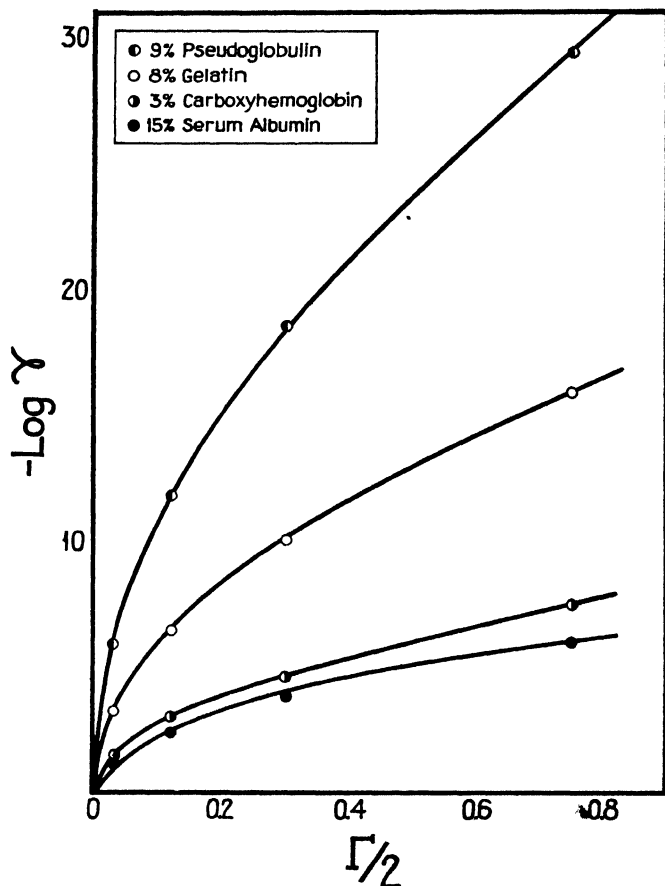


FIG. 2. The influence of calcium chloride on the activity coefficients of various proteins at 25°, as estimated from the effects of the proteins on the activity of the salt.

where γ_2 and γ_2^0 are, respectively, protein activity coefficients in the presence of salt and in pure water. The solubilities of proteins in the presence of salts have been described by equations analogous to Equation 4 (3, 8, 19).

According to Ferry, Cohn, and Newman (5), this is an empirically valid method of characterizing solubility data. Although their results on the solubility of hemoglobin in ethanol-water mixtures can be characterized by some form of Equation 4, they consider it of greater theoretical significance to express their results in the form given by the Kirkwood theory (11) for the electrostatic interaction of ions and complex dipoles. This, as they have shown, leads to a linear relation at very low concentrations of salt, but approximates a square root formulation at higher concentrations. Their treatment involves also the consideration of non-electrostatic forces. Accordingly in Table VII, $-\log \gamma_2/\gamma_2^0$ is given as a function of μ , the ionic strength, which for the systems under consideration is approximately equal to $\Gamma/2$, the concentration unit by which Ferry, Cohn, and Newman describe their results. These results are given graphically in Fig. 2, which illustrates the effect of calcium chloride on the activity coefficients of the proteins.

DISCUSSION

In all the systems studied, the effect of the salt is to reduce the activity coefficient of the protein, according to the empirical Equation 4. The magnitude of this effect varies widely for different proteins, as solubility studies have also indicated, increasing in the order, carboxyhemoglobin, serum albumin, gelatin, pseudoglobulin. The calculated results for carboxyhemoglobin indicate effects of the same order of magnitude as those obtained by solubility studies on this protein (5, 8, 22). They are, however, based on E.M.F. of about 1 millivolt, and are not of great significance. The small E.M.F.'s obtained with this protein are related to its low solubility, and correspond to its negligible effect on the osmotic coefficient of sodium chloride as determined by the freezing point method (21). The results for the other proteins are more significant, since for all of them potentials of at least 3 or 4 millivolts could be obtained.

That there is a correspondence between the activity coefficients and other electrostatic properties of the proteins is indicated by consideration of the values of the molal dielectric increments of the proteins. The molal dielectric increment δ is approximately 22,000 for carboxyhemoglobin (18), 20,000 for the most polar

serum albumin fraction studied by Ferry and Oncley (6), and 135,000 for the pseudoglobulin fraction employed in the present studies.¹ The value of δ for gelatin is of the order of 90,000 (4). Thus, there appears to be a rough parallel between the molal dielectric increments of the proteins and their interaction with neutral salts. The relation between these two effects has been thoroughly discussed by Cohn (2) for the case of amino acids and peptides. The results of the present study appear to indicate a general parallelism between δ and the salt effects, even for such highly complex molecules as the proteins.

The author wishes to express his appreciation to Professor Edwin J. Cohn for his interest in this work, and to Professor George Scatchard for his criticisms of the manuscript.

SUMMARY

1. A new type of amalgam electrode for studying the interaction of proteins and neutral salts is described. Decomposition of the amalgam by protein is prevented by a cellophane membrane, which is equilibrated with the solution.

2. It is shown theoretically that such a membrane, provided it be in equilibrium with the solution, introduces no significant potential into the cell.

3. The E.M.F.'s obtained with this electrode are shown to agree with those obtained with flowing amalgam electrodes.

4. The effects of several proteins on the activity of calcium chloride and other salts have been determined.

5. From the potentiometric data, the effects of calcium chloride and the other salts on the activity coefficients have been determined. These effects are comparable with the results of solubility studies.

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PURIFICATION OF CRYSTALLOIDS AND COLLOIDS BY ELECTRODIALYSIS

By NORMAN R. JOSEPH

(From the Department of Physical Chemistry, Harvard Medical School, Boston)

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Preparations of amino acids, peptides, and other substances of a similar character often contain appreciable quantities of inorganic salts and other electrolytic impurities which are usually very difficult to remove by recrystallization. This difficulty results from the fact that the solubility relations of the substances involved are often very similar with respect to various solvents. In most cases several recrystallizations are necessary to prepare electrolyte-free amino acids or peptides, and this usually results in considerable losses of material.

To surmount this difficulty a simple electrodialytic technique has been devised, suggested by the Adair-Keys method for the determination of total base (1). As illustrated in Fig. 1, the apparatus consists of a beaker which contains the solution to be purified and two cylinders which serve as anode and cathode chambers. These may be of any convenient dimensions, depending on the volume of solution to be purified and the concentration of electrolyte to be removed. The lower end of each cylinder is closed by a cellophane membrane. Cellophane, previously moistened and blotted, is stretched tightly over the end of the cylinder and fixed to the glass by means of collodion. About 1 cc. of mercury is introduced into each cylinder. This is covered by a small volume of dilute HCl. Platinum electrodes make contact with the acid solutions. In the electrodialysis, a 110 volt direct current is employed, passing through an external resistance of 1000 ohms. Anions in the impure solution migrate to the mercury anode and collect within the membrane as insoluble salts of mercury, such as calomel, mercurous sulfate, etc. Traces of these substances generally appear in the solution,

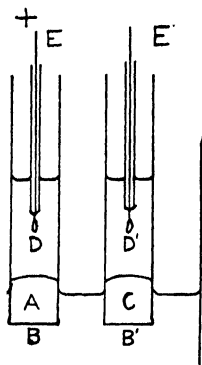


FIG. 1. *A*, mercury anode; *B*, *B'*, cellophane membranes; *C*, mercury cathode; *D*, *D'*, dilute acid solutions; *E*, *E'*, platinum electrodes.

TABLE I

Purification of Various Substances by Electrodialysis

Volume, approximately 25 cc.

Material and approximate concentration	Initial conductivity	Time of electro-dialysis	Conductivity
	<i>reciprocal ohms</i> $\times 10^6$	<i>hrs.</i>	<i>reciprocal ohms</i> $\times 10^6$
Distilled water	4.4	6	1.4
Technical glycine, 0.5 M*	82.7	3	8.1
“ “ + 0.001 M KCl, 0.5 M*	223	5	7.9
“ “ + 0.001 M KCl, 0.5 M*	223	1	16.2
“ “ + 0.001 M KCl, 0.5 M*	223	2	12.3
“ “ + 0.001 M KCl, 0.5 M*	223	4	7.7
“ “ + 0.001 M KCl, 0.5 M*	223	6	7.6
“ “ + 0.001 M (NH ₄) ₂ SO ₄ , 0.5 M*	355	4	8.6
“ “ + 0.001 M (NH ₄) ₂ SO ₄ , 0.5 M*	355	6	8.2
β -Alanine, 0.5 M*	1595	4	505
“ “ + 0.001 M (NH ₄) ₂ SO ₄ , 0.5 M*	1595	20	24.5
“ “ + 0.001 M (NH ₄) ₂ SO ₄ , 0.5 M*	1595	24	23.0
Carboxyhemoglobin, 1.5%†	650	12	84
“ “ + 0.001 M (NH ₄) ₂ SO ₄ , 0.5 M*	650	24	45
“ “ + 0.001 M (NH ₄) ₂ SO ₄ , 0.5 M*	650	30	45

* The final concentration agrees with the initial within a few per cent; checked by formol titration.

† Electrodesialyzed at 5°.

but are readily removed by filtration or centrifugation. Cations such as sodium, potassium, ammonium, etc., form amalgams within the cathode cylinder, and these react with the acid above the mercury, displacing hydrogen. Formation of gas bubbles in the acid solutions within the anode and cathode chambers is an effective indication of the conductivity of the solution that is being purified. Electrodialysis is continued until the evolution of gas in these chambers is very slow. It is then discontinued and the conductivity of the sample is determined. The process is continued until a limiting value is obtained.

The results of several electrodialyses of various solutions are presented in Table I.

It is evident from these results that crystalloids containing a high percentage of electrolytic impurities can be very effectively purified within a few hours by electrodialysis. The conductivity of the glycine solutions purified by this method is slightly lower than the values reported by Mehl and Schmidt (2) for glycine which had been recrystallized several times. It should be pointed out that this technique can also be applied to the purification of colloids as well as crystalloids. The carboxyhemoglobin solution electrodialyzed by this method showed no indication of methemoglobin on spectroscopic examination.

SUMMARY

1. A method for the purification of crystalloids by electrodialysis is described. The anode and cathode are mercury within cellophane membranes.

2. Results are given for the electrodialysis of some impure amino acid solutions and for a solution of carboxyhemoglobin.

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THE INFLUENCE OF BILE ON EROSIONS OF THE CHICK GIZZARD LINING

BY H. J. ALMQUIST AND E. MECCHI

(From the Division of Poultry Husbandry, College of Agriculture,
University of California, Berkeley)

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Evidence on the necessity of an organic dietary factor for the maintenance of the chick gizzard lining has been provided in a number of papers (1-4). Progress in the isolation of an anti-gizzard erosion factor has been reported by Bird and Oleson (5) who attribute a protective action against erosions to chondroitin fractions from cartilage. Almquist (6) has found that whole bile or bile acids, particularly cholic acid, were effective dietary supplements in preventing or curing erosions of the gizzard lining. These studies of the effect of bile acids on gizzard erosions have been extended.

Methods and Results

The diet used in this work consisted of ether-extracted sardine meal 17.5, ether-extracted dried brewers' yeast 7.5, salt plus small amounts of manganese, copper, and iron 1.0, cod liver oil 1.0, and ground polished rice 73.0 parts. A hexane extract of alfalfa, treated with activated magnesium oxide and filtered, was added to provide adequate vitamin K. On this diet chicks grew at normal rates without regard to gizzard erosions. The diet was evidently complete in all respects except for the gizzard factor.

Chicks were kept in electrically heated, metal battery brooders with wire floors. They were maintained for approximately 2 weeks on the basal diet and then were given the supplemented diets for approximately 3 weeks.

The average gizzard erosion score (1, 2) was determined by arbitrary visual scoring of each gizzard lining on the basis of 0

score for no erosion, 0.5 for a doubtful case of erosion, 1 for a definite erosion, and 2 for a severe erosion. In a few cases a score of 3, indicating a very severe and wide-spread erosion, was assigned. The sum of the individual scores was then divided by the number of chicks in the group.

TABLE I

Comparative Effects of Bile Acids on Erosions and on Cholic Acid of Gizzard Linings and of Gallbladder Bile

Supplement to basal diet	Per cent of diet	No. of chicks	Average gizzard erosion score*	Cholic acid content	
				Dried gizzard lining	Dried gall-bladder bile
				mg. per gm.	mg. per gm.
None		10	1.25	2.1	
"		10	1.10	2.5	
"		10	1.30	2.4	122
"		10	1.05	3.0	97
Cholic acid	0.5	10	0.00		
" "	0.5	10	0.20	14.7	
" "	0.5	11	0.14	12.0	
" "	0.5	10	0.15	14.0	480
Desoxycholic acid†	0.5	8	0.60		
" " †	0.5	12	0.83	2.0	
" " †	0.5	9	1.00	3.0	87
Dehydrocholic acid§	0.5	10	0.20	1.6	
" " §	0.5	10	0.25	4.1	84
" " §, 		9	0.77	1.7	

* 0 = no erosion; 0.5 = doubtful erosion; 1 = definite erosion; 2 = severe erosion.

† Contained approximately 7 per cent cholic acid.

‡ Contained approximately 1.8 per cent cholic acid.

§ Contained no cholic acid.

|| Given by five intramuscular (breast) injections of a 10 per cent aqueous solution of the sodium salt in total quantity equivalent to 0.5 per cent of the diet consumed.

Cholic acid in the gizzard lining was determined quantitatively by a reasonably accurate procedure. After removal from the chicks, the gizzards and linings were cut open, thoroughly washed, and then allowed to stand in water for several hours. The linings were peeled from the gizzards, dried in a current of air at

room temperature, and then finely ground. The ground lining was extracted several times with 95 per cent ethanol at room temperature to remove any free bile acids, then placed in a Soxhlet extractor and extracted for 15 hours with hot 95 per cent ethanol to which had been added 2 per cent of concentrated hydrochloric acid. Cholic acid in the acid-alcohol extract was estimated by an adaptation of the specific colorimetric furfural procedure (7). In a few instances the cholic acid content of the gallbladder bile was also determined. The results of these experiments are given in Table I.

DISCUSSION

It is quite evident from a comparison of the average gizzard erosion scores that cholic acid is a much more effective anti-erosion supplement than desoxycholic acid. Dehydrocholic acid, a synthetic bile acid, is more effective in the diet than in the form of a sodium salt given by intramuscular injection. The general stimulating effect of all these substances on the secretion of bile bears no apparent relation to their power to cure or prevent gizzard erosions. The comparative ineffectiveness of desoxycholic acid and of injected dehydrocholic acid seems to indicate that the mechanism of the curative action of cholic and dehydrocholic acids on the gizzard lining is a local one. Injection experiments with other bile acids proved impractical because of their marked toxicity when so administered.

The curative effect of cholic acid appears to involve its combination with the gizzard lining. When desoxycholic acid or no supplement at all was fed, the eroded condition of the linings was quite severe, and the cholic acid contents of the linings were all very similar and small. Cholic acid as a dietary supplement greatly increased the cholic acid content of the lining, at the same time curing the erosions. On the other hand, the synthetic, unnatural dehydrocholic acid did not significantly alter the cholic acid content of the lining as compared to that of the groups on the basal diet, although it likewise exerted a strong curative influence on erosions. This may mean that dehydrocholic acid functions in the same way as cholic acid in curing erosions.

Determination of cholic acid in the portion of lining lost by erosion was, of course, impossible, and it may be that in the case

of severely eroded linings the data given for the remainder of the lining are somewhat too high, since the portion of the lining which remains sound is likely to retain a normal content of cholic acid.

In certain experiments the contents of the gallbladders were collected according to groups. Chicks fed only the basal diet had very small gallbladders from which, in many individuals, scarcely any bile could be obtained. Chicks fed any of the bile acid supplements had normal gallbladders which yielded good quantities of bile. It was apparent that chicks on the basal diet were comparatively poorly supplied with bile, a condition which is consistent with the eroded state of their gizzard linings. Regardless of the differences in volume of bile per chick, the total cholic acid per gm. of dried gallbladder bile was slightly lower in the groups fed dehydrocholic acid and desoxycholic acid than in the basal groups. Cholic acid in the diet caused a large increase in the total bile cholic acid.

At this point may be added some further observations having a bearing on the subject. Gizzard linings of chicks fed only the basal diet have a normal yellow color even when the diet is free of fat-soluble plant pigments. Colorimetric reactions of the alcohol-extracted pigments from the gizzard lining indicate that they are bile pigments. Cholic acid in the diet causes a marked decrease in the alcohol-extractable pigments of the gizzard lining, while desoxycholic and dehydrocholic acids cause, if anything, a slight increase.

These facts suggest that bile normally, at least at intervals, finds its way into the gizzard.¹ When the bile is deficient in cholic acid or when the quantity of bile is small, erosions of the lining develop but the coloration of the lining by bile pigments is not diminished. On the other hand, when cholic acid from the diet is constantly present, the erosions are cured, but the normal entrance of bile may be inhibited and the coloration thereby diminished.

If these assumptions are correct, then desoxycholic and dehydrocholic acids have no similar inhibiting effect on the presence of bile in the gizzard. The slight curative action of desoxycholic

¹ Dr. L. W. Taylor has called our attention to the fact, frequently observed by him, that the gizzard of the chick embryo may, in the later stages, contain bile as identified by the characteristic tests for bile pigments.

acid might, therefore, be attributed to its known power as a cholagogue. The marked curative action of dehydrocholic acid cannot be adequately explained in this way, and it is necessary to assume direct action as well.

The presence of gizzard erosions in chicks on the basal diet seems to be related principally to the small quantity of bile. The basal diet evidently lacks some property or substance which leads to the normal secretion of bile. Conceivably, then, any condition, such as disease, malnutrition, or poor environment, which might inhibit the normal secretion of bile, directly or indirectly, may lead to or enhance a condition of gizzard erosion. This is a possible explanation for many cases of gizzard erosion that occur in commercial flocks of chicks, and which seem, frequently, to be associated with poor development. The presence of gizzard erosions in chicks has often been accepted as sufficient cause of an unthriftiness; however, it can be shown, experimentally, that growth and development are not noticeably hindered by severe erosions. The occurrence of gizzard erosions in commercial chicks is probably to be regarded as an *effect* of rather than a *cause* of poor growth and health.

The authors are indebted to Professor C. L. A. Schmidt for his interest and advice, and to the Works Progress Administration, Project A. P. No. 465-03-3-209, for general assistance in this work. We are grateful to Riedel-de Haen, Inc., for provision of certain of the bile acids.

SUMMARY

Cholic acid is an effective dietary supplement in the cure or prevention of erosions of the gizzard lining. Dehydrocholic acid is almost equally effective, while desoxycholic acid is comparatively ineffective.

Cholic acid is an integral part of the gizzard lining and a deficiency of bile or of cholic acid in the chick leads to erosions of the lining.

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A METHOD FOR THE ESTIMATION OF ULTRAMICRO-QUANTITIES OF LACTIC ACID

By BENJAMIN F. MILLER AND JOHN A. MUNTZ

(From the Walter G. Zoller Memorial Dental Clinic and the Department of Medicine of the University of Chicago, Chicago)

(Received for publication, August 11, 1938)

This method was developed to evaluate the rôle of lactic acid in the pathogenesis of human dental caries. Lactic acid has long been considered the most important factor in the dissolution of the mineral material which occurs in dental caries; however, experimental proof for this assumption has been lacking. To study this problem it is desirable to investigate the presence or absence of lactic acid in the very early carious lesions. These areas contain such minute quantities of soluble material that existing methods are inadequate for the determination of the amounts of lactic acid which may be present.

This report describes a quantitative method for lactic acid based upon the highly sensitive color reaction of Eegriwe (1933). Eegriwe's qualitative test utilizes the oxidation of lactic acid to acetaldehyde by hot, concentrated sulfuric acid; from the acetaldehyde an intense, bluish violet color is produced by reaction with *p*-hydroxydiphenyl. By a thorough study of the factors influencing the oxidation of lactic acid, and the subsequent color reaction, we have been able to convert the Eegriwe test into a method for the quantitative estimation of ultramicroquantities of lactic acid.

All the methods hitherto available¹ (except that of Kirk)² require 10 to 100 micrograms of lactic acid. Our method has an

¹ The various current methods for quantitative estimation of lactic acid in biological materials are reviewed by Peters and Van Slyke (1932).

² Dr. P. L. Kirk has kindly sent us details of his unpublished method for the ultramicrodetermination of lactic acid by a distillation and titration procedure. In its present form Dr. Kirk's method is somewhat less sensitive than the procedure given in this paper, and it is considerably more time-consuming.

optimum sensitivity between 2 and 10 micrograms, and may be employed to determine quantities as small as 0.1 microgram. The method has certain other advantages over existing ones in that (1) it is almost completely specific for lactic acid,³ (2) it does not give a reaction with carbohydrates, and thereby obviates the procedures for removal of carbohydrate, and (3) it is extremely simple to perform, and its actual working time per determination appears to be less than that of any other method.

As shown later the method is very well suited to the analysis of lactic acid in carious tooth material. Because of the several advantages of the method over existing ones, it seemed desirable to extend it to determination of lactic acid in biological fluids such as serum and saliva.

Reagents

Sulfuric acid, sp. gr. 1.84 (Kahlbaum's *pro analysi* grade).

p-Hydroxydiphenyl (Eastman). This compound must be purified as follows: Dissolve about 30 gm. in a minimum volume of warm, pure acetone. Add water until a solid begins to separate and chill the solution to 0°. Remove the crystals by filtration; redissolve them in acetone and repeat the crystallization twice. The third crop of crystals is dried rather completely by a current of clean air, and then dissolved in a minimum volume of hot absolute acetone (about 55°). The solution is chilled to 0°; the crystals are filtered off and dried for 12 hours at 80° in a perfectly clean place.

Metaphosphoric acid (Reagent grade). A 7 per cent aqueous solution is prepared fresh each day.

Procedure

Exactly 0.2 cc. of the sample is measured into a glass-stoppered, Pyrex test-tube (15 × 120 mm.). The tube is placed in ice water while exactly 1.5 cc. of the concentrated sulfuric acid are added

³ Dr. E. S. G. Barron (personal communication) employs an enzyme found in the gonococcus (Barron and Hastings, 1933) to oxidize lactic acid to pyruvic acid, and determines lactic acid by measuring the oxygen uptake in the Warburg apparatus. The method is specific for α -hydroxy acids. It requires a sample of lactic acid about 100 times greater than our method.

slowly. The ground glass portion of the tube is lubricated conveniently with the small amount of acid that remains in the tip of the 1.5 cc. delivery pipette. The tube is stoppered tightly, and its contents are mixed by gentle shaking. The tube is then placed in a copper rack and heated in a boiling water bath for 5 minutes. It is cooled immediately in deep ice water for 10 to 15 minutes and then removed from the bath and gently rotated for a few seconds on its long axis at an angle of about 25° to the horizontal. It is placed in the upright position, allowed to drain for a few seconds, and then unstoppered. 8 mg. of very finely pulverized *p*-hydroxydiphenyl⁴ (weighed to within 0.2 mg.) are added through a small funnel whose partially constricted stem almost touches the solution in the tube. This technique prevents undesirable scattering of the powder on the walls of the test-tube. The tube is restoppered firmly and its contents mixed by gentle, but thorough agitation. The mixture should not be splashed on the walls of the tube.

The tube is kept at room temperature for 1 hour to develop maximum color. It is then placed in a boiling water bath for exactly 90 seconds and cooled immediately to room temperature. After this final heating the solution should have a clear, blue-violet color. The color is stable for several hours at room temperature.

The solutions are read against a blank in a Pulfrich stufen-photometer at 5 mm. stratum length and through the S-57 filter. The 5 mm. cup is supported on a small pad because of the small volume of fluid employed; with such a pad the diaphragm aperture is adequately covered even though the cup contains only 1.5 to 1.7 cc. of fluid. The extinction coefficient is obtained and its value converted to micrograms of lactic acid per 0.2 cc. sample by means of a calibration chart.

The blank determination is made by subjecting 0.2 cc. samples of H_2O to the entire procedure described above. The extinction coefficient of the blank should be less than 0.025.

⁴ *p*-Hydroxydiphenyl is used in the solid form because attempts to find a non-reactive solvent were unsuccessful. The following solvents dissolve the reagent but all interfere with the method: ethyl alcohol, methyl alcohol, *n*-butyl alcohol, isoamyl alcohol, carbon tetrachloride, chloroform, acetone, dioxane, and ether.

Determination of Lactate in Teeth and Carious Material—The tooth or carious material is pulverized in a mortar, weighed, and extracted by shaking for 30 minutes in a suitable volume of redistilled water (1.5 cc. is most convenient). The supernatant fluid is filtered through a sintered glass filter which has been coated with acid-washed asbestos. 0.2 cc. of the clear filtrate is used for the analysis.

Determination of Lactate in Serum—To 1 volume of blood serum are added 1 volume of fresh 7 per cent metaphosphoric acid and 8 volumes of H_2O . After thorough agitation, the mixture is allowed to stand for 10 minutes, and then centrifuged at high speed (3500 R.P.M.) for 10 minutes. The supernatant fluid, with suitable dilution if necessary, is analyzed in aliquots containing 0.2 cc. Removal of carbohydrate is unnecessary. Whenever possible, filtrates should be diluted to contain 2 to 10 micrograms of lactic acid in 0.2 cc.

Determination of Lactate in Saliva—1 volume of saliva is diluted with an equal volume of H_2O . 0.2 cc. samples are analyzed by the usual procedure.

Modified Procedure for Specific Determination of Lactic Acid in Presence of Pyruvic Acid—The method given above allows 5 minutes for sulfuric acid to oxidize the lactic acid to acetaldehyde. With this procedure, pyruvic acid has been found to yield a color equivalent to 20 per cent of that given by an equal weight of lactic acid. We have found that longer periods of heating with sulfuric acid diminish the color given by pyruvic acid, and after 15 minutes heating the color reaction with pyruvic acid disappears completely. With the longer period of heating the final color given by lactic acid is only about 8 per cent less than that obtained in the shorter period with the regular procedure. Thus, if necessary, lactic acid may be sharply differentiated from pyruvic acid by increasing the period of heating with sulfuric acid to 15 minutes. The other details of the procedure remain unaltered. If the modified method is employed, a new calibration should be obtained with standard lactate solutions.

EXPERIMENTAL

Calibration of the Method—As noted in the section on procedure the concentration of lactic acid is obtained from a graph relating the extinction coefficient to concentration of lactic acid. The

calibration is made on standard solutions containing pure lithium lactate,⁵ in concentrations varying from 0.5 to 10 micrograms per 0.2 cc. (The concentrations refer to the lactic acid equivalent of lithium lactate.) The extinction coefficients are plotted against the concentration of lactic acid on linear graph paper (or the per cent light transmission may be charted against concentration on semilogarithmic paper). A linear relationship between extinction coefficient and concentration of lactate is found from 0.5 to 10 micrograms per 0.2 cc. of sample, indicating that the light absorption in this range obeys the Lambert-Beer law.

Sensitivity and Precision of the Determination in Pure Solutions of Lithium Lactate—The calibration line as determined above has been checked at different times on fresh lactate solutions and is reproducible to within 5 per cent. Duplicate analyses on quantities of from 3 to 10 micrograms check within 2 to 3 per cent, and with 1 microgram per sample agree within 5 per cent. If an unusually high degree of precision is required, *e. g.* 2 per cent in the range of 0.5 to 1.5 micrograms per sample, it is advisable to determine several standard lactate solutions of concentrations corresponding to the unknown solutions.

The extreme sensitivity of the color reaction is demonstrated by the span of 75 per cent in light transmission for a change in concentration of from 1 to 10 micrograms. It should be noted that the determination is performed at the shortest stratum length ordinarily employed in the Pulfrich stufenphotometer. For semiquantitative measurements on amounts of lactic acid between 0.1 and 0.5 microgram per sample we have employed a 50 mm. stratum length cup and a special microattachment which is interchangeable with the cup holders ordinarily used in the stufenphotometer. This special cup requires only 1 cc. at a 50 mm. stratum length. However, in our experience it is much more convenient to use the 5 mm. macrocup whenever possible.

The Pulfrich stufenphotometer has been employed because of its availability in our laboratory; undoubtedly the microattachment of the Evelyn photoelectric colorimeter (1936) could be used satisfactorily. For analyses requiring less precision one may employ the Duboscq colorimeter with microcups.

Specificity—The method has been tested on a number of organic

⁵ Lithium lactate was prepared by the method of Hillig (1937).

acids which are related structurally to lactic acid, or which may be associated with lactic acid in biological reactions. In addition a few non-acidic compounds of similar structure to lactic acid have been studied. The reactions are given in Table I, which shows that the method has a very high degree of specificity for lactic acid as compared with other compounds. It should be noted that

TABLE I

Comparison of Color Reaction Given by Lactic Acid and Related Compounds

In each test 6 micrograms of the compounds listed below were used.

Compounds giving blank test	Reactive compounds	
Malic acid	β -Rhamnose	Very slight trace of color
Fumaric acid	1,3-Dihydroxybutyric	
Malonic acid	lactone	
Succinic acid		
Citric acid	Pyruvic acid (5 min. period of heating with H_2SO_4)	Color equal to that given by 1 microgram lactic acid
Tartaric acid		
Glycerophosphoric acid		
Acetic acid		
Glycolic acid	Pyruvic acid (15 min. period of heating with H_2SO_4)	Blank test
Oxalic acid		
Formic acid		
2,3-Dihydroxybutyric acid		
β -Hydroxy butyrate		
2,3-Dihydroxybutyric lactone		
α -Hydroxyisobutyric acid		
α -Hydroxy- <i>n</i> -butyric acid		

the slight color given by pyruvic acid can be removed entirely by use of the modified method described above.

Analysis of Lactate in Powdered Tooth Substance—To test the effect of carious tooth substance on the determination of lactic acid the following experiment was performed: 200 mg. of carious tooth powder were eluted with 5 cc. of cold water. The suspension was filtered through asbestos, and 0.2 cc. of the filtrate was evaporated

TABLE II

Analysis of Lactate Added to Aqueous Extract of Carious Tooth Material

Lactic acid added	Lactic acid found	Error
micrograms	micrograms per 0.2 cc.	micrograms per 0.2 cc.
0	1.0	
0.5	1.3	-0.2
1.0	1.75	-0.25
3.0	3.92	-0.08
4.0	4.75	-0.25
5.0	6.10	+0.1
6.0	7.3	+0.3
8.0	9.05	+0.05
10.0	10.85	-0.15

TABLE III

Determination of Lactic Acid in Tooth Substance, Serum, and Saliva Containing Added Lactate

Material analyzed	Lactic acid originally present	Lactic acid added*	Lactic acid found	Error
	mg. per cent	mg. per cent	mg. per cent	per cent
Serum	39.5	48.0	89.0	+1.7
"	21.2	22.2	45.3	+4.4
"	21.2	26.6	49.8	+4.2
	micrograms per 0.2 cc.	micrograms	micrograms per 0.2 cc.	
Carious tooth elution	0.4	5.0	5.6	+3.7
" " "	0.5	5.0	5.6	+1.8
" " "	0.3	5.0	5.6	+5.4
	micrograms			
" " "	2	75	75.4	-2.1
" " "	22.8	50	74.0	+2.3
" " "	115.5	25	138.6	-1.3
	mg. per cent	mg. per cent	mg. per cent	
Saliva	4.9	4.0	8.2	-7.9
"	3.2	5.0	7.9	-3.7
"	3.1	4.0	6.9	-2.8

* The lactic acid was added as lithium lactate. The values in the table are all calculated as lactic acid.

just to dryness at 80°. To the residue in each tube 0.2 cc. of standard lactate solution was added and the lactic acid determined in the usual manner. Suitable controls were performed without added lactate. In Table II the lactic acid found by analysis is compared with the calculated concentrations. It is shown there that aqueous extracts of carious tooth substance do not interfere with the sensitivity of the method. Further examples of the accuracy of the method in the presence of tooth extracts are given in Table III.

Accuracy of Method When Applied to Determination of Lactic Acid in Serum and Saliva—Data showing the satisfactory recovery

TABLE IV

Simultaneous Determinations of Lactic Acid in Serum by the Avery-Hastings Gasometric Method and the Authors' Ultramicromethod

Human serum sample No.	Lactic acid found by Avery-Hastings method	Lactic acid found by ultramicromethod*
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	23.4	22.9
2	18.8	19.1
3	39.7	41.8
4†	89.0	91.2
5†	62.0	60.1

* The ultramicroanalyses were performed on 1:10 metaphosphoric acid filtrates of serum.

† These sera contained added lactate.

of lactic acid from serum and saliva are given in Table III. The results of analyses performed simultaneously on sera by the gasometric method of Avery and Hastings (1931-32) and by the authors' method are presented in Table IV. The two methods give almost identical values.

SUMMARY

A method for the precise determination of ultramicroquantities of lactic acid in carious tooth substance, serum, and saliva has been described. The method has its optimum sensitivity in the range of 2 to 10 micrograms, and it may be employed to estimate quantities as small as 0.1 microgram of lactic acid. The advan-

tages of the method over current macro- and micromethods for lactic acid are its increased sensitivity and specificity, elimination of procedures for removal of carbohydrates, and its simplicity and brevity of operation.

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RIBONUCLEODEPOLYMERASE (THE JONES-DUBOS ENZYME)

BY GERHARD SCHMIDT AND P. A. LEVENE

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

(Received for publication, August 1, 1938)

Earlier observations in this laboratory¹ have led to the view that the dephosphorylation of polynucleotides is preceded by their disintegration into nucleic acids of simpler order and that the action of specific enzymes, the polynucleotidases, is responsible for this preliminary disintegration. Although both enzyme complexes (polynucleotidase and nucleophosphatase) have been found to be present in intestinal juice, attempts to separate them have so far been beset by great difficulties. Especially it was not possible to obtain from intestinal juice a sufficiently active polynucleotidase free from nucleophosphatase. It therefore seemed more promising to continue studies on polynucleotidase with extracts from organs which are poorer in nucleophosphatase than is the small intestine.

In the present paper our observations on the effect of pancreatin on yeast nucleic acid are described. Jones² discovered in 1920 the presence in the pancreas of a thermostable enzyme capable of hydrolyzing yeast nucleic acid without a release of either phosphoric acid or bases. In a second paper³ he reported the isolation of the four mononucleotides from the digested solution. Recently, Dubos and Dubos and Thompson^{4,5} have confirmed the observations of Jones to the extent of proving the presence in pancreas gland of a thermostable enzyme bringing about a cleavage

¹ Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, **88**, 753 (1930); **96**, 461 (1932).

² Jones, W., *Am. J. Physiol.*, **52**, 203 (1920).

³ Jones, W., and Perkins, M. E., *J. Biol. Chem.*, **55**, 557 (1923).

⁴ Dubos, R. J., *Science*, **85**, 549 (1937).

⁵ Dubos, R. J., and Thompson, R. H. S., *J. Biol. Chem.*, **124**, 501 (1938).

of ribonucleic acid. They have also achieved a certain degree of purification of the enzyme. However, they did not enter into an analysis of the chemical changes brought about by this enzyme.

The present experiments are in agreement with those of Dubos and Thompson, confirming the view of Jones that the degradation of yeast nucleic acid in presence of heat-treated pancreas extract is due to the effect of an enzyme. It has now been demonstrated that nucleic acid itself is not altered at the pH chosen for the digestion with pancreatin. Furthermore, the rate of degradation depends on the *amount* of pancreatin used.

Dr. J. H. Northrop and Dr. M. Kunitz informed us that the trypsin-inhibiting fraction obtained from pancreas gland contained an enzyme similar to that of Jones and Dubos. Dr. G. M. Meyer of this laboratory prepared the fraction for us and, indeed, it proved to contain the enzyme in a higher degree of concentration than the solutions obtained from pancreatin. The action of this enzyme was so rapid that it was used for a lecture demonstration, inasmuch as a nucleic acid solution which formed an abundant precipitate with glacial acetic acid remained perfectly clear with this precipitant if digested with the enzyme for 5 minutes only.

Contrary to the observation of Jones, the action of the enzyme solution heated according to the directions of Dubos and Thompson was limited to depolymerization without the formation of mononucleotides. It is possible that Jones was dealing with a more complex enzyme solution, inasmuch as his starting material was fresh pancreas gland whereas our enzyme was derived from commercial pancreatin.

In our experiments the entire product of digestion could be separated into two fractions; one was precipitable with hydrochloric acid, while the larger part (about 60 per cent) was obtainable from the neutralized filtrate as an insoluble barium salt. Both fractions contained the ratios N/P and (purine N)/(total N) required by a tetranucleotide of ribonucleic acid ($N/P = 1.7$; (purine N)/(total N) = 0.66; molar ratio of guanine to adenine = 1:1). They differ from yeast nucleic acid and from each other in their higher solubility in hydrochloric and glacial acetic acids.

It is clear that each fraction represents a mixture of tetranucleotides in different stages of polymerization. The absence of appreciable amounts of mononucleotides in these fractions was

demonstrated, first, by the observation that the products of digestion of yeast nucleic acid with boiled pancreatin are not dialyzable through cellophane, while a mixture of the four *mononucleotides* (obtained by alkaline hydrolysis of yeast nucleic acid, under the conditions of Steudel and Peiser⁶) passes through this membrane very rapidly; second, no appreciable change in depression of the freezing point of the digested mixture was observed during the digestion of yeast nucleic acid with boiled pancreatin, while under similar conditions, a considerable depression was observed after alkaline hydrolysis of ribonucleic acid. (The formation by alkaline hydrolysis of cleavage products lower than mononucleotides can be excluded on the grounds of the absence of reducing power (negative Fehling's test) and of inorganic phosphoric acid in the hydrolysate.)

CONCLUSIONS

1. The presence in the pancreas gland of a heat-stable nuclease, as recorded first by Jones and later by Dubos, is confirmed.
2. The function of the enzyme is that of a depolymerizing agent, limited to the dissociation of the tetranucleotides of high molecular weight into those of lower molecular weight.
3. Native ribonucleic acid is a polymer of the tetranucleotide.

EXPERIMENTAL

Methods

Purification of Yeast Nucleic Acid—Commercial yeast nucleic acid (Boehringer) was purified according to Levene⁷ by means of glacial acetic acid.

The purified nucleic acid did not change its composition after frequent extractions with water, fractional reprecipitation with glacial acetic acid, or reprecipitation with hydrochloric acid-alcohol.

Determination of Yeast Nucleic Acid—The precipitation of yeast nucleic acid with hydrochloric acid or with glacial acetic acid was found the most satisfactory method for its evaluation. Thus, from a 10 per cent solution of nucleic acid there was re-

⁶ Steudel, H., and Peiser, E., *Z. physiol. Chem.*, **120**, 292 (1922).

⁷ Levene, P. A., *Biochem. Z.*, **17**, 120 (1909).

covered by means of hydrochloric acid 93 per cent; by glacial acetic acid 96 per cent; from a 2 per cent solution 85 and 87 per cent respectively; and from a 0.5 per cent solution 80 and 83 per cent respectively.

The amount of nucleic acid recovered is practically constant at a given concentration and is not influenced by the presence of the pancreatin extracts used in this investigation. Glacial acetic acid offers some advantages over hydrochloric acid for precipitation of ribonucleic acid, since it forms a flocculent precipitate which can be easily washed on the centrifuge. The conditions for the optimal precipitation of yeast nucleic acid are as follows:

With Glacial Acetic Acid—To 1 volume of approximately neutral nucleic acid solution (the concentration should be at least 1 per cent) are added 10 volumes of glacial acetic acid with stirring. After 20 minutes, the precipitate is centrifuged off and washed once with glacial acetic acid.

With Hydrochloric Acid—0.2 cc. of concentrated hydrochloric acid is added drop by drop to 5 cc. of the neutral nucleic acid solution in a centrifuge tube. The oily precipitate very soon settles to the bottom and is centrifuged off immediately. The precipitate is washed once with 5 cc. of dilute hydrochloric acid (0.2 cc. of concentrated hydrochloric acid in 5 cc. of water). In the presence of tissue extracts, clear filtrates are obtained.

Determination of Total Phosphorus in Nucleic Acid Precipitates—After combustion of the precipitate according to Kirk,⁸ the phosphorus was determined by Embden's⁹ original method. (The modifications introduced by Kirk, useful as they are for the manometric determination, cannot be applied in the gravimetric method.) For the first washing of the strychnine phosphomolybdate, 0.7 per cent nitric acid, as recommended by Kirk, was used.

Microdetermination of Purines—25 to 50 mg. of substance were hydrolyzed with 10 cc. of 2 per cent sulfuric acid. The purine bases were precipitated with silver sulfate, the precipitates washed with silver sulfate solution (not with water), decomposed with hydrochloric acid, and the nitrogen determination made by the micro-Kjeldahl method.

The precipitation of the purines with silver sulfate is complete;

⁸ Kirk, E., *J. Biol. Chem.*, **106**, 191 (1934).

⁹ Embden, G., *Z. physiol. Chem.*, **113**, 138 (1921).

in comparison with Krüger and Schmid's copper reagent,¹⁰ the silver method has the advantage that the adsorption of nitrogen-containing impurities is practically negligible and that the purines can be completely recovered from their silver compounds.

Enzymes—The enzyme solutions were always prepared according to the method of Dubos. For some experiments, the solution was purified by dialysis for 12 to 16 hours against distilled water, with subsequent concentration of the dialyzed solution under reduced pressure to one-fifth of its original volume and precipitation with an excess of barium hydroxide solution. After 10 minutes, the precipitate was centrifuged off and the solution neutralized with 5 N and (towards the end) with 1.0 N sulfuric acid. After centrifuging, the supernatant solution was used for the experiments. The principal purpose of this purification was to remove from the enzyme solution substances which would interfere with the isolation of the products of the enzymatic hydrolysis of the nucleic acid.

Stability of Yeast Nucleic Acid under the pH Conditions of the Enzyme Experiments

2 per cent solutions of sodium yeast nucleinate were incubated at pH 6.7 and pH 8.4 (time 16 hours, temperature 37°). The nucleic acid could be recovered quantitatively in both samples and the pH remained practically unchanged during the incubation.

Influence of Amount of Pancreatin on Rate of Hydrolysis

Three samples (5 cc. each) of 10 per cent yeast nucleinate solution were incubated during 16 hours at 37° with different amounts (Table I) of the Dubos solution. The total volume of each incubated solution = 25 cc.; pH = 6.7. After the incubation, the nucleic acid P was determined, the results being given in Table I.

Time Curve of Enzyme Action—10 cc. of 10 per cent yeast nucleinate solution (warmed to 37°) were mixed with 100 cc. of dialyzed Dubos solution (warmed to 37°) and the mixture (pH 6.7) incubated at 37° during various intervals of time.

¹⁰ Schmidt, G., *Z. physiol. Chem.*, **219**, 191 (1933). Krüger, M., and Schmid, J., *Z. physiol. Chem.*, **45**, 7 (1905).

At different intervals, samples (10 cc.) were pipetted off for the determination of nucleic acid. The results are given in Fig. 1.

TABLE I
Effect of Enzyme Concentration on Extent of Hydrolysis

Amount of Dubos solution	Unchanged nucleic acid recovered after digestion	
	Phosphorus	Nucleic acid
cc.	mg.	per cent
0	37.9	100
1	31.5	83.1
5	14.7	38.7
20	11.6	30.5

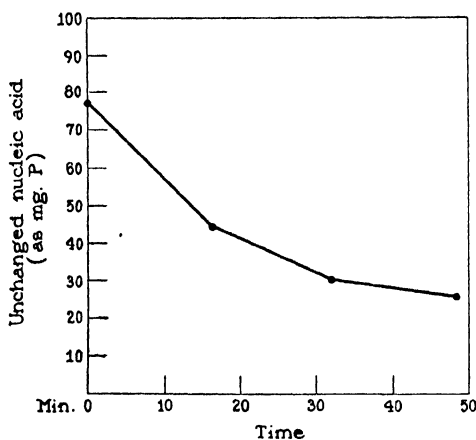


FIG. 1. Rate of enzyme action

Nature of Degradation Products of Yeast Nucleic Acid during Digestion with Heated Pancreatin

Freezing Point after Digestion with Enzyme—50 cc. of a 10 per cent yeast nucleic acid solution were digested with 250 cc. of a dialyzed Dubos solution (20 hours, 37°). After concentration under diminished pressure, the solution was diluted to a volume of 50 cc. and the freezing point was determined. Found, $\Delta_f = -1.85^\circ$.

Control—The same amounts of nucleic acid and enzyme solu-

tions were incubated *separately* (20 hours, 37°), concentrated, mixed, and the mixture diluted to a volume of 50 cc. The freezing point was determined immediately afterwards. Found, $\Delta_F = -1.85^\circ$. The increase of Δ_F after enzymatic hydrolysis was, $\Delta_{\Delta_F} = 0$.

Freezing Point after Digestion with NaOH—50 cc. of a 10 per cent solution of sodium yeast nucleinate were cooled to 0° and mixed with 5 cc. of a 33 per cent solution of sodium hydroxide. Freezing point determinations were made immediately and after 12 and 36 hours standing of the alkaline nucleinate solution at room temperature. The values found for Δ_F after 0, 12, and 36 hours standing were -2.29° , -2.57° , and -2.65° , respectively.

TABLE II
Total Phosphorus in Bag after Various Time Intervals

Time of dialysis	Total P content in bag		
	Incubated with water (a)	Incubated with yeast nucleic acid (b)	(b) - (a)
hrs.	mg.	mg.	mg.
0	89.6	657.0	567.4
6	79.7	683.8	604.1
8	69.5	646.6	577.1
14	53.2	617.5	564.3
19	36.8	592.8	556.0

The increase of Δ_F after alkaline hydrolysis during 36 hours was $\Delta_{\Delta_F} = -0.36^\circ$.

Dialysis Experiments. Non-Dialyzability of Digestion Products—500 cc. of enzyme solution (corresponding to 150 gm. of pancreatin and purified by precipitation with barium hydroxide) were incubated with 100 cc. of a 10 per cent solution of sodium yeast nucleinate (12 hours, 37°, pH 6.7). After the incubation, only 31.8 per cent of the nucleic acid remained precipitable with glacial acetic acid.

Another portion of the enzyme solution was incubated with water under the same conditions.

Each of the digested solutions was concentrated under reduced pressure to a volume of 160 cc. and then dialyzed during 19 hours. The values of the total phosphorus in the bag, at different stages of the dialysis, are given in Table II. The last column records the

difference between the corresponding values in the bags with and without the digested yeast nucleic acid.

Dialyzability of Mononucleotides—10 gm. of yeast nucleic acid were hydrolyzed according to Steudel and Peiser.⁶

This mixture was neutralized by means of acetic acid and dialyzed under the conditions described above. After dialysis during 4 hours, only 36.5 per cent of the initial nucleic acid P remained in the bag; after 12 hours, all but 8.6 per cent of the nucleic acid P had passed through the cellophane membrane.

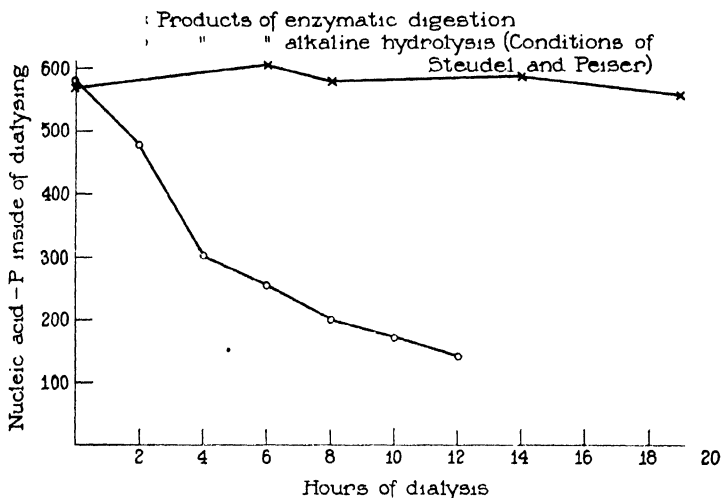


FIG. 2. Dialysis of mononucleotides

The results of both dialysis experiments are shown graphically in Fig. 2.

Fractionation of Digestion Products—For the time being, the fractionation was limited to the separation of the product of enzymatic action into two parts; one *insoluble* in hydrochloric acid (Fraction I), the other *soluble* (Fraction II).

Fraction II could be precipitated almost quantitatively by means of barium acetate in 30 per cent aqueous methyl alcohol solution. No attempt has so far been made to obtain further fractionation.

Composition of Fraction I. Preparation—20 gm. of yeast nu-

cleinate were digested with 1000 cc. of purified Dubos solution (see above) (corresponding to 200 gm. of pancreatin, Parke, Davis) until the mixture yielded only a slight precipitate with glacial acetic acid. Incubation at 37° during a period of 12 to 24 hours was usually sufficient to reach this stage of digestion. The solution was then concentrated under reduced pressure to a volume of 150 to 200 cc. During the concentration the acidity was observed several times and adjusted to neutrality towards litmus paper by means of sodium hydroxide or acetic acid. To the cooled concentrate, a 25 per cent hydrochloric acid solution was added in small portions until the precipitation was complete. The precipitate was immediately centrifuged off, redissolved by being suspended in water and neutralized with sodium hydroxide, and reprecipitated by the addition of hydrochloric acid. The sticky precipitate was quickly washed in the centrifuge with 0.5 N hydrochloric acid and then ground up and carefully washed with 95 per cent alcohol. Finally, it was left overnight under absolute alcohol and then washed with absolute alcohol and dry ether.

The product was analyzed for total N, purine N, and P. In addition, the purine bases were isolated according to the method of Levene.^{11, 12}

Analysis of Fraction I. Total N—5.681 mg. of substance (dried at 100°) yielded 0.666 cc. of N₂ (28°, 762 mm.). Found, N 13.33 per cent.

Purine N—The silver precipitate, obtained from the hydrolysate of 72.5 mg. of Fraction I was analyzed for nitrogen by the Kjeldahl method. 4.68 cc. of 0.1 N H₂SO₄ were consumed. Found, purine N 9.1 per cent.

Total P—An aqueous solution of the ash from 95.5 mg. of substance (dried at 100°) was diluted to 100 cc. 5 cc. of this solution yielded 33.8 mg. of strychnine phosphomolybdate. Found, P 7.92 per cent.

Isolation of Purine Bases—From the hydrolysate of 2.755 gm. of Fraction I, 290 mg. of crude guanine and 680 mg. of adenine picrate were isolated. The guanine had the following composition.

3.038 mg. substance: 1.235 cc. N₂ (27° and 761 mm.)

C₅H₄ON₅. Calculated, N 46.35; found, N 46.04

¹¹ Levene, P. A., *J. Biol. Chem.*, **53**, 441 (1922).

¹² Levene, P. A., and Jorpes, E., *J. Biol. Chem.*, **86**, 389 (1930).

The adenine picrate was recrystallized once from water. It had the following composition.

3.012 mg. substance: 0.820 cc. N₂ (27° and 753 mm.)

C₈H₈N₅·C₆H₃O₇N₃. Calculated, N 30.76; found, N 30.68

From the above figures, the following ratios may be calculated.

Yeast nucleic acid.	Calculated.	$\frac{N}{P} = 1.69,$	$\frac{\text{purine } N}{\text{total } N} = 0.67$
	Found.	$\frac{N}{P} = 1.72,$	$\frac{\text{purine } N}{\text{total } N} = 0.68$

Comparison of Solubilities of Fraction I and Yeast Nucleic Acid in Hydrochloric Acid and Acetic Acid—Solutions containing the neutral sodium salts of yeast nucleic acid or the neutral salt of Fraction I, in various concentrations, were precipitated (a) by addition of 0.04 volume of concentrated hydrochloric acid, (b) by addition of 5 volumes of glacial acetic acid, (c) by addition of 10 volumes of glacial acetic acid. The total phosphorus content of the precipitates was then determined.

The results of the experiments with yeast nucleic acid are shown in Fig. 3; the results with Fraction I, in Fig. 4. The abscissæ represent the concentration of the solution, the ordinates the values of the total phosphorus of the precipitates. Each figure contains three curves, Curve A representing the results with hydrochloric acid, Curve B with 5 volumes, and Curve C with 10 volumes of glacial acetic acid.

Fraction II—The supernatant liquor from Fraction I was rendered alkaline (pH 8) with sodium hydroxide. After addition of one-third of its volume of absolute methyl alcohol, the solution was precipitated with a slight excess of barium acetate solution in 33 per cent aqueous methyl alcohol. The precipitate was washed with 33 per cent aqueous methyl alcohol, redissolved by suspending in water and acidifying with the minimum of concentrated hydrochloric acid, and reprecipitated by neutralization with baryta water with subsequent addition of one-third of its volume of methyl alcohol and of a solution of barium acetate in methyl alcohol.

The barium salt was washed with 30 per cent methyl alcohol and finally with absolute ethyl alcohol and dry ether.

Analysis of Fraction II. Total N—5.300 mg. of substance yielded 0.504 cc. of N_2 at 29° and 760 mm. Found, N 10.74 per cent.

Purine N—The silver precipitate, obtained from the hydrolysate of 402 mg. of Fraction II was analyzed for nitrogen by the Kjel-

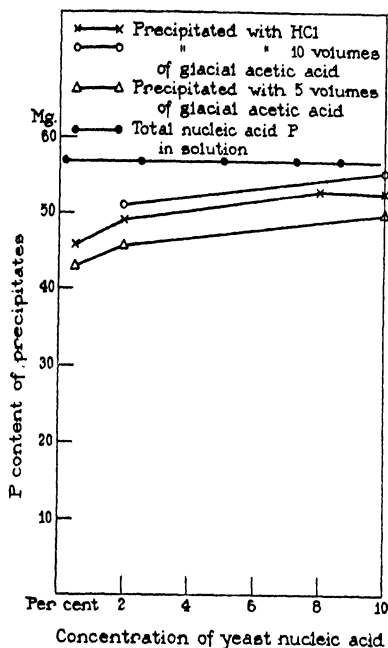


FIG. 3

FIG. 3. Precipitability of yeast nucleic acid.

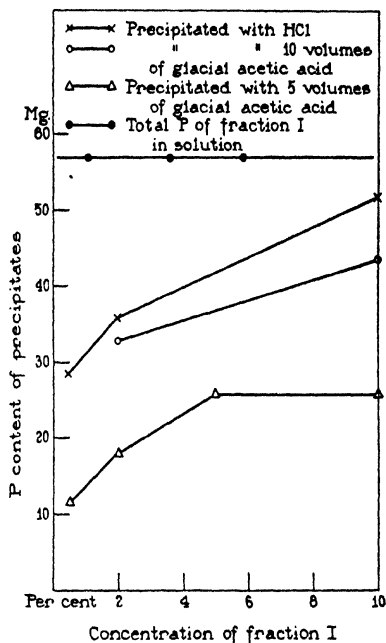


FIG. 4

FIG. 4. Precipitability of Fraction I.

dahl method. 19.1 cc. of 0.1 N H_2SO_4 were consumed. Found, N 6.65 per cent.

Total P—The ash solution of 47.6 mg. was brought to a volume of 100 cc. 25 cc. of this solution yielded 69.6 mg. of strychnine phosphomolybdate. Found, P 6.55 per cent.

Ba—4.02 gm. of substance yielded 1.2736 gm. of $BaSO_4$. Found, Ba 17.78 per cent.

Isolation of Purine Bases—From the hydrolysate of 3.48 gm. of

Fraction II, 300 mg. of crude guanine and 500 mg. of adenine picrate were isolated.

Identification of Purine Bases—The guanine was purified in the usual way.

3.175 mg. substance: 1.289 cc. N₂ (28° and 759 mm.)

C₅H₅ON₅. Calculated, N 46.35; found, N 45.95

The adenine picrate was recrystallized twice.

3.592 mg. substance: 0.963 cc. N₂ (26° and 764 mm.)

C₈H₆N₆·C₈H₃O₇N₃. Calculated, N 30.76; found, N 30.75

From the above figures, the following ratios result (the numbers in parentheses represent the values as calculated for yeast nucleic acid).

$$\frac{N}{P} = 1.64 \text{ (1.69)}, \quad \frac{\text{purine N}}{\text{total N}} = 0.62 \text{ (0.66)}$$

$$\text{Molar ratio,} \quad \frac{\text{adenine}}{\text{total purine bases}} = 0.45 \text{ (0.50)}$$

$$\text{“ “} \quad \frac{\text{guanine}}{\text{total purine bases}} = 0.52 \text{ (0.50)}$$

THE DIFFUSION OF TOBACCO MOSAIC VIRUS PROTEIN IN AQUEOUS SOLUTION

BY HANS NEURATH AND ARTHUR M. SAUM

(From the Department of Chemistry, Cornell University, Ithaca)

(Received for publication, June 22, 1938)

From a knowledge of the sedimentation constant and the diffusion constant, the molecular weight of a large number of proteins has been determined by means of the following relationship (1),

$$M = \frac{RTs}{(1 - V\rho)D} \quad (1)$$

where M is the molecular weight, s the sedimentation constant, V the partial specific volume of the solute, and D the diffusion constant.

For elongated particles, the molecular weight can be estimated from diffusion data and viscosity data alone, with an equation derived by Perrin (2),

$$\frac{D}{D^0} = \frac{\sqrt[3]{a^2/b^2}}{\sqrt{1 - (a^2/b^2)}} \ln \frac{1 + \sqrt{1 - (a^2/b^2)}}{a/b} \quad (2)$$

where D is the observed diffusion constant, D^0 the diffusion constant on the assumption of spherical molecules, and a/b the ratio of short to long axis of the ellipsoid of revolution. The ratio of the axes can, as a first approximation, be obtained from viscosity measurements of dilute protein solutions, with Kuhn's equation (3).

$$\frac{\eta}{\eta_0} - 1 = G \left(2.5 + (b/a)^2 \frac{1}{16} \right) \quad (3)$$

Here η/η_0 is the relative viscosity and G the concentration of the solute in volume per cent.¹

¹ The general validity of this equation is open to various objections. Guth (4) derived, on the basis of a prolate ellipsoid, an equation which becomes identical with Kuhn's equation if $b \gg a$.

Measurements of the sedimentation constant of tobacco mosaic virus protein have been reported by Eriksson-Quensel and Svedberg (5) and by Wyckoff (6-8) and probable values of the relative dimensions of this protein have been computed recently from viscosity data (9). In order to determine the molecular weight from either of the above equations (Equations 1 and 2) the diffusion constant must be known. We have carried out diffusion measurements with preparations of tobacco mosaic virus protein, kindly placed at our disposal by Dr. W. M. Stanley. This paper describes results of such measurements by the refractometric method of Lamm (10).

Material and Methods

Tobacco mosaic virus protein, dissolved in 0.1 M phosphate buffer at pH 6.8, was prepared by the chemical method of Stanley (11). The solutions showed streaming double refraction and remained practically stable even after 4 months storage at 2°. Before each measurement, the protein solutions were allowed to dialyze for several days at 2° against 0.1 M phosphate buffer through cellophane membranes, the dialysate being used as solvent for the diffusion process.

The diffusion apparatus was built in this laboratory according to the specifications given by Lamm (10) with but slight modifications. The diffusion cell was of stainless steel with plane parallel windows, and the slide was made of lucite. The lens of the camera and the windows of the thermostat were of quartz. The green band of a mercury arc served as light source and light filters consisted of Corning filters No. 351 and No. 512 and of a solution of copper chloride in methanol. The photographic plates were Eastman Kodak spectroscopic plates GV, sensitized for the green. The photographic enlargement factor was 1.201, the aperture of the lens 60.0 cm. The scale was a mm. scale subdivided into 0.5 mm. in the region near the center of the cell. All measurements refer to $25^{\circ} \pm 0.002^{\circ}$. A detailed account of the diffusion apparatus will be given in a subsequent publication.

Results

The displacement of each scale line was plotted against the position of the displaced line in the usual manner and the diffusion constants calculated with the following equation (10).

$$D = \frac{\eta}{\eta_0} \frac{\sigma^2}{2t} \left(\frac{l-b}{l} \right)^2 \quad (4)$$

where D = diffusion constant in sq. cm. per second, η = viscosity of solvent, η_0 = viscosity of water, t = time elapsed in seconds, $((l-b)/l)^2 = 0.8985$. The uniformity of the dispersion was studied by the statistical method described by Lamm (10), which consists of comparing the normal dispersion curve with the experimental one in normal coordinates.

TABLE I

Results of Diffusion Measurements of Tobacco Mosaic Virus Protein

$\Sigma(S)$ = summation over all ordinates, $X_{\max.}$ = maximum ordinate in normal coordinates, D = diffusion constant in sq. cm. per second.

Experiment No.	Protein concentration	Time	$\Sigma(S)$	$X_{\max.}$	D
	<i>per cent</i>	<i>sec.</i>	10^3		10^8
1	0.2*	77,220	252.2		2.93
		141,780	255.9		2.74
2	0.5	94,260	305.1	0.456	3.13
		160,140	322.3	0.448	2.98
		325,800	332.4		2.92
3	1.0	431,580	378.7	0.500	2.00
		526,500	391.0		2.12
		594,540	400.2	0.510	2.08
4	1.5	525,900	554.5	0.458	2.39
		837,420	572.9	0.488	2.38

* The accuracy of this experiment was somewhat inhibited by the small displacements of the scale lines in this low protein concentration.

Table I gives the results of diffusion measurements in four different concentrations of virus protein. Figs. 1 and 2 illustrate two typical diffusion curves in normal coordinates.

Inspection of Table I and Figs. 1 and 2 reveals a considerable deviation from the ideal dispersion curve in respect to both the maximal displacement and symmetry. It has been shown that chemically prepared tobacco mosaic is polydisperse (5, 6) and that ultracentrifugally prepared virus protein becomes polydisperse if placed in phosphate buffer and allowed to stand (7). Recent ultracentrifugal studies suggest that this polydispersity is brought about by contact of the virus protein with salt, since in the absence of electrolytes only one particle size could be detected (7). Inas-

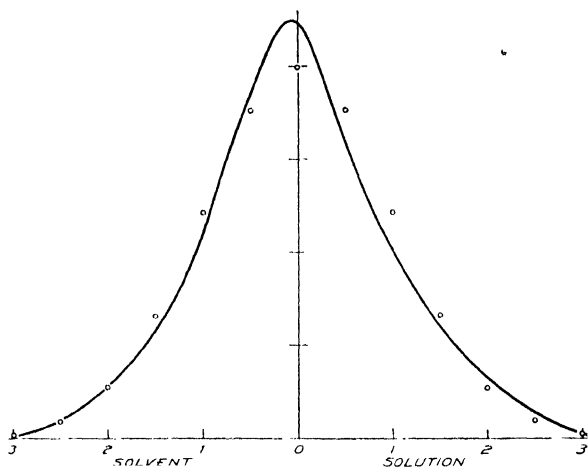


FIG. 1. Diffusion curve and ideal distribution points of 0.5 per cent virus protein in normal coordinates. The abscissa represents the distance from the boundary; ordinate, displacement of scale lines.

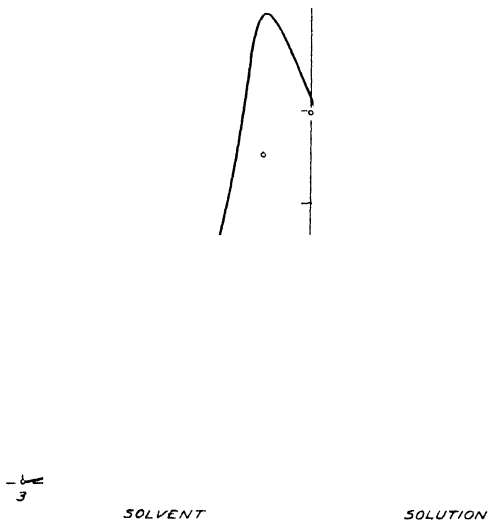


FIG. 2. Diffusion curve and ideal distribution points of 1 per cent virus protein in normal coordinates. The abscissa represents the distance from the boundary; ordinate, displacement of scale lines.

much as our measurements were carried out with chemically prepared virus protein in the presence of phosphate buffer, it is reasonable to assume that the anomalous maximum displacement may be partly due to polydispersity.

The independence of the diffusion constant, D , of time, as well as the slight variations of Σ (S) with time, justifies the conclusion that, under the given experimental conditions, the diffusion process proceeds in a normal way. Hence we can regard the values of D as expressing average values of the diffusion constant of all particle species present.²

The asymmetric shape of the diffusion curves, the shift of the maximum ordinate towards the side of the solvent, and possibly also the anomalous maximum displacement are apparently due to an acceleration of the rate of diffusion as the particles migrate from

TABLE II
Average Distance between Virus Molecules in Relation to Concentration

Concentration	Distance
per cent	10^6 cm.
1.5	1.77
1.0	2.02
0.5	2.55
0.2	3.46
0.05	5.50

the solution into the solvent. In many instances, similar effects have been ascribed to a charge effect. There is, however, but little likelihood that in the presence of 0.1 M phosphate buffer a diffusion potential arises in spite of the protein being negatively charged at pH 6.8.³

Mutual hindrance of solute particles from free diffusion may be considered as a more important factor responsible for anomalous diffusion. From an average particle weight of 50,000,000 (9),

² Apparently this method of calculation allows one to compute average diffusion constants in the case of uniform and also non-uniform diffusion. The calculation of D from two ordinates of the curve would fail however, as the special composition of the solution varies along the x direction (10).

³ Usually diffusion potentials of colloidal solutions can be considered to become negligible if the conductivity of the electrolytes present in the solution is about 50 times as high as that of the colloidal material.

we have calculated the mean interparticle distance for the various concentrations of virus protein used in this research, and have arrived at the values given in Table II.

In a previous communication (9), the length of the major axis of rod-shaped virus particles has been estimated and found to be about 4×10^{-5} cm. This value is decidedly larger than the average interparticle distance in the range of concentration investigated and it appears to be very likely that the observed deviations from the ideal diffusion process are due to mutual interference between the solute particles. A comparison between the observed diffusion constant and the concentration reveals indeed that, within the limits of the experimental error, the diffusion constant is highest for lowest protein concentrations. Likewise, the experimental diffusion curve approaches the ideal curve with increasing dilution.

DISCUSSION

Taking 3×10^{-8} as the average value of the diffusion constant of our material and 0.73 as the partial specific volume (12), we have calculated the molecular weight of tobacco mosaic virus protein with the aid of Equation 1. Eriksson-Quensel and Svedberg (5) reported an average sedimentation constant of 235×10^{-13} for polydisperse solutions of chemically prepared virus protein. Using more carefully prepared material, Wyckoff (6) found the presence of two molecular components⁴ with sedimentation constants of 174×10^{-13} and about 200×10^{-13} . It was only in certain cases that aggregation increased to such an extent as to produce a sedimentation constant of about 235×10^{-13} (8). Using the value of $s = 200 \times 10^{-13}$, we calculated a molecular weight of 68,400,000 (or 59,000,000, if the lower sedimentation constant is used). As our solutions had been standing longer than 2 months before the diffusion experiments, we are inclined to attach more significance to the higher value.

It has been reported that a stable, monodisperse solution of this protein can be obtained only in the absence of electrolytes (7). We have attempted to carry out diffusion measurements

⁴ Chemically prepared virus proteins showed the presence of a single molecular component ($s = 174 \times 10^{-13}$) only if the protein was extracted from the plants shortly after inoculation (8).

with a sample of tobacco mosaic virus protein prepared by repeated ultracentrifugation of infected juice, using distilled water as solvent. Preliminary experiments failed, however, owing to the formation of a yellow, tenuous film at the boundary between solvent and solution 24 hours after the diffusion cell had been placed in the thermostat.⁵ No further experiments with this preparation were performed, as it was to be expected that in the absence of electrolytes a diffusion potential would arise at pH 6 (the isoelectric point of this protein is about pH 3.5 (5)).

It is very probable that the diffusion constant of monodisperse solutions does not differ greatly from that of our solutions, and, as a first approximation, we can consider the calculated value of 59,000,000 to be the molecular weight of monodisperse tobacco mosaic virus protein. The value of 17,000,000, previously reported (5), is definitely too low and was based on an arbitrary dissymmetry constant of 1.3.

In addition we have calculated the molecular weight using Perrin's equation which, as stated, relates the diffusion constant to the shape of the molecules (14). Assuming a value of b/a of 37 (9), we obtained a value of $M = 90,000,000$. This discrepancy we attribute to the very elongated shape of virus molecules. Such anisotropic molecules will show an orientation in the streaming liquid (13) parallel to the direction of flow, whereas the distribution will be random in the liquid at rest. Hence the observed viscosity and the ratio of the axes calculated from it are probably lower than those which actually obtain. This would explain the high molecular weight calculated from viscosity and diffusion data. The molecular weight of 42,600,000 calculated from the sedimentation constant and viscosity data (9) may for the same reason be regarded as too low. This conclusion emerges from the fact that the relative viscosity of monodisperse virus protein solutions is the same as that of polydisperse solutions, when measured with an Ostwald viscosimeter (9). It should be different, however, as partial aggregation of molecules would change their average length to width ratio. Viscosity measurements under very low hydrostatic pressures may confirm our considerations.

⁵ In addition we found that separation into two optically different layers, as described previously (12, 13), occurred during dialysis of this material against distilled water, even in concentrations as low as 4 mg. per cc.

In order to get an idea of the true shape of tobacco mosaic virus protein, we have calculated its dissymmetry constant from the sedimentation constant and the molecular weight (1). For $M = 59,000,000$ and $s = 174 \times 10^{-13}$ the dissymmetry constant is 3.12, whereas it is 3.00 if the respective values for the heavier component are used. The ratio of the axes, calculated from these values by Perrin's equation, is 1:52 and 1:56 respectively. This would, on the assumption of a prolate ellipsoid, correspond to a particle of about 14 $m\mu$ in diameter and 720 $m\mu$ in length.

SUMMARY

Diffusion measurements of tobacco mosaic virus protein, prepared by the chemical method of Stanley, and dissolved in 0.1 M phosphate buffer, have been carried out by means of the refractometric method of Lamm. The observed diffusion constant is about 3×10^{-8} sq. cm. per second in the most dilute solutions. The average molecular weights calculated from this value and the sedimentation constants are about 64,800,000 for the heavier component and about 59,000,000 for the normal component. The ratio of the axes, computed from these values, is about 1:55.

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THE VISCOSITY OF TOBACCO MOSAIC VIRUS PROTEIN SOLUTIONS

By MAX A. LAUFFER

*(From the Department of Animal and Plant Pathology of The Rockefeller
Institute for Medical Research, Princeton)*

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It is well known that the viscosity of a liquid is a measure of its resistance to shear. Upon the introduction of small solid objects into a viscous medium, the internal motion of the liquid when caused to flow is made more complicated than it would be otherwise and the resistance to shear is thereby increased. Einstein (4) has derived the following formula relating the viscosity of a suspension of solid spheres (η) to the viscosity of the solvent (η_0) and the fraction of the total volume occupied by the spheres (G).

$$\frac{\eta}{\eta_0} - 1 = 2.5G \quad (1)$$

If the spherical particles have electrical charges, the internal motion of the liquid will be complicated still further and the increase in viscosity will be greater than that given by the Einstein equation. Von Smoluchowski (19) has formulated the following relationship, taking into account this additional effect.

$$\frac{\eta}{\eta_0} - 1 = 2.5G \left(1 + \left(\frac{\zeta D}{2\pi} \right)^2 \frac{R}{\eta_0 r^2} \right) \quad (2)$$

where ζ is the electrokinetic potential, D is the dielectric constant of the medium, R is the specific electrical resistance, and r is the radius of the spheres.

The contribution to the internal friction of a liquid made by a rod-like particle oriented perpendicular to the direction of motion of the liquid is greater than that of a sphere of equal volume. However, the contribution of a rod-shaped particle oriented paral-

lel to the direction of motion is less than when oriented perpendicularly. Kuhn (11) has derived the following equation for the viscosity of a suspension of randomly oriented rod-like particles.

$$\frac{\eta}{\eta_0} - 1 = 2.5G + \frac{G}{1.5} \left(\frac{b}{a}\right)^2 \quad (3)$$

where b/a is the ratio of length to diameter of the particles. The model which Kuhn used to approximate a rod consisted of a number of rigidly joined spheres. Guth (9) derived the same equation for a rod-like model consisting of an ellipsoid of revolution. An alternate equation has been derived by Eizenschitz (6) for a model consisting of an ellipsoid of revolution, which reduces to the following form for particles in which the length greatly exceeds the diameter.

$$\frac{\eta}{\eta_0} - 1 = G \frac{(b/a)^2}{15(\ln 2(b/a) - (3/2))} \quad (4)$$

The unsimplified form of this equation reduces to the Einstein equation just as does the Kuhn-Guth equation for particles in which $b/a = 1$. If one assumes that the particles are completely oriented by the streaming liquid, the contribution to the viscosity of the liquid will be much less and will be given, according to Eizenschitz (5), by the following equation.

$$\frac{\eta}{\eta_0} - 1 = \frac{1.15}{\pi} G \frac{(b/a)}{\ln 2(b/a)} \quad (5)$$

It has been established that the molecules of the tobacco mosaic virus protein are rod-shaped (1-3, 13, 14, 22). Studies on the viscosity of solutions of the protein have been reported by Stanley (20), by Frampton and Neurath (8), and by Laufer (12). The interpretation of such viscosity data is seen to be fraught with a considerable degree of uncertainty. If it is assumed that the rod-like particles are not appreciably charged (see Equation 2), that they are not appreciably oriented in the stream of the viscometer, and that they are not appreciably hydrated, it is permissible to apply the Kuhn-Guth or Eizenschitz equations (Equation 3 or 4). It is a known fact that the rod-like particles of the tobacco mosaic virus protein do orient somewhat in a flowing stream, for the

material shows stream double refraction under such circumstances. Furthermore, from the results of Mehl (15), one would expect a considerable degree of orientation of particles in a viscous system with a velocity gradient of the order encountered in a capillary viscometer. However, complete orientation should not be encountered in the rod-like particles of tobacco mosaic virus protein because of the finite thickness of the particles and because of the effect of Brownian movement. When either Equation 3 or Equation 4 is used, therefore, it must be remembered that the asymmetry calculated should be too small.

Presentation and Discussion of Results

Viscosity As Function of Concentration—The viscosities reported in this study were measured, with a high precision quartz viscometer,¹ on very dilute solutions of several independently isolated samples of the protein prepared by ultracentrifugation repeated four or five times. The kinetic energy correction was applied in those few cases in which it was appreciable. In Table I² are given the results of measurements of the specific viscosity of the tobacco mosaic virus protein dissolved in water at various concentrations. A linear relationship is seen to hold between specific viscosity and concentration in the lowest concentration ranges, showing that there is no interaction between particles at those concentrations. This linearity does not hold for solutions as concentrated as 1 per cent. Neurath and Saum (17) found anomalous diffusion for tobacco mosaic virus protein at a concentration of 1 per cent, which they explained as being due to interaction between the particles.

Interpreted by means of Equation 3, the results of Table I indicate a ratio of particle length to thickness of about 35:1, and

¹ The author wishes to express his gratitude to Dr. D. A. MacInnes and Dr. L. G. Longworth for the use of the quartz viscometer and the facilities of their laboratory.

² Although the data found in this study indicate that the specific viscosity of ultracentrifugally isolated tobacco mosaic virus protein is a constant property from preparation to preparation, results obtained in another connection seem to indicate that the specific viscosity is somewhat dependent upon the treatment given the protein during its isolation. This observation may explain the difference between the results of Stanley (20) and those presented in this paper.

with use of Equation 4 this value turns out to be about 63. With knowledge of the shape of the tobacco mosaic virus protein particles, the sedimentation studies reported by Eriksson-Quensel and Svedberg (7) and by Wyckoff (24, 26) may be interpreted somewhat more fully than was previously possible. An equation which may take the following form has been derived, expressing the dissymmetry factor used by Svedberg (21), f/f_0 , as a function

TABLE I

Specific Viscosities of Aqueous Solutions of Tobacco Mosaic Virus Protein at 20°

$\frac{\eta}{\eta_0} - 1$	C	$\frac{(\eta/\eta_0) - 1}{C}$
	<i>gm. protein per cc. solution</i>	
0.0059	0.000099	59.60
0.0165	0.000296	55.74
0.0272	0.000458	59.39
0.0278	0.000494	56.28
0.0542	0.000920	58.91
0.0566	0.000988	57.29
0.6009	0.009200	65.25

of the ratio of the major to the minor axes of the colloidal particles regarded as being rod-like ellipsoids of revolution (10, 18).

$$\frac{f_0}{f} = \frac{(a/b)^{\frac{1}{2}}}{\sqrt{1 - (a/b)^2}} \log_e \frac{1 + \sqrt{1 - (a/b)^2}}{a/b} \quad (6)$$

Once this dissymmetry factor is known, the molecular weight of the suspended particles may be calculated with the equation (21).

$$\frac{f}{f_0} = \frac{M(1 - Vd)}{6\pi\eta_0 N s_{20} (3MV/4\pi N)^{\frac{1}{2}}} \quad (7)$$

M is the molecular weight, V is the partial specific volume of the protein taken to be 0.73 (1, 20), d is the density of the solvent, N is Avogadro's constant, and s_{20} is the sedimentation constant taken to be 174×10^{-13} (24). With the value for b/a obtained from Equation 3, a molecular weight of 42.6×10^6 is obtained for the tobacco mosaic virus protein. This corresponds to particles 12.3 $m\mu$ in diameter and 430 $m\mu$ in length. With the value of b/a obtained from Equation 4, a value for M of 63.2×10^6 ,

corresponding to particles 11.5 $m\mu$ in diameter and 725 $m\mu$ in length, is obtained. Both of these sets of values are of the same order of magnitude as those determined by diffusion (8, 16, 17), stream double refraction (15), ultrafiltration (1, 23), and x-ray diffraction (2, 3) studies.

Even though no high degree of reliability can be ascribed to the results of any of the methods just considered, since the agreement obtained in the results of the several independent methods is fairly good, it seems reasonable to assume that the size and shape of the tobacco mosaic virus protein particles actually are of the order of those indicated by these studies. As a working model, the tobacco mosaic virus protein molecule may be regarded as being a cylindrical body, 12.3 $m\mu$ in diameter and 430 $m\mu$ in length, having a molecular weight of 42.6×10^6 . With this model it is possible to interpret the rather puzzling observation that chemically isolated preparations of the protein and ultracentrifugally isolated preparations which have remained in contact with electrolytes show two sedimentation boundaries in the ultracentrifuge, one with a sedimentation constant of about 174×10^{-13} and the other with a constant of 200×10^{-13} (24). The most carefully prepared protein has a single constant of 174×10^{-13} . The simplest way to dispose of this second boundary is to regard it as being due to a second component formed by the association of the particles represented by the slower moving boundary. If 2 rod-shaped molecules having the dimensions of the working model from a supply having a sedimentation constant of 174×10^{-13} associate end to end, they will contribute to a second supply having a molecular weight of 85×10^6 and a ratio of particle length to diameter of 70. With Equations 6 and 7, it can be shown that such a supply would have a sedimentation constant of 202×10^{-13} , a figure in very good agreement with the experimental value of 200×10^{-13} . Similar calculations made with a particle having the dimensions found from Equation 4 as a model would yield a value for the sedimentation constant of the second component somewhat under the observed one. Some model intermediate between the two would yield the exact value. This good agreement between calculated and observed sedimentation constants of the second component is evidence in favor of the essential correctness of the model chosen, as well as for the explanation of the origin of the second boundary.

By combining Equations 6 and 7 and by assuming, as was done above, that the faster moving boundary represents particles formed by the end to end association of two particles from the slower moving supply, it is possible to write two simultaneous equations involving only M and b/a of the original particles as variables. Hence M and b/a may be evaluated from the sedimentation constants of the two species present in double boundaried preparations. Wyckoff (25) has given the sedimentation constants of double boundaried preparations of the protein of aucuba mosaic virus, a strain of tobacco mosaic virus, as 185×10^{-13} and 220×10^{-13} . According to the method here described, the molecular weight of the slower component of the aucuba mosaic virus protein is found to be about 32.5×10^6 , corresponding to particles with a length of $267 \text{ m}\mu$ and a diameter of $13.7 \text{ m}\mu$. The molecular weight of the second component would be about 65×10^6 , corresponding to particles $534 \text{ m}\mu$ long and $13.7 \text{ m}\mu$ in diameter. It should be emphasized that the nature of the assumptions made in this calculation cause the molecular weight derived from it to be a minimum value, for, if any type of association other than a perfect end to end type is assumed, the values obtained for the molecular weight and the dissymmetry of the original molecules will be greater than those obtained in this case.

In a manner similar to that used in the calculation of the sedimentation constant of the heavy component of tobacco mosaic virus protein, it can be shown that, if the tobacco mosaic virus protein represented by the sedimentation constant of 174×10^{-13} is to be regarded as being in an associated state itself, as some workers believe, then the maximum sedimentation constant which could possibly be shown by the hypothetical original molecules (particles one-half as long but of the same diameter as the model) would be 145×10^{-13} . No evidence from the ultracentrifuge for any active component having such a sedimentation constant or a lower one has as yet been reported, even in studies on unpurified juice from diseased tobacco plants (26).

Viscosity As Function of pH—In Table II are presented the results of viscosity measurements on solutions of the protein in buffers of ionic strength of 0.02 at various values of pH. These measurements were carried out on solutions containing about 0.5 mg. of protein per cc. It is seen that in the alkaline range the

viscosity falls off gradually as pH increases. In the range between pH 5.5 and about 7, the specific viscosity is essentially constant, but it rises sharply between 5.5 and 4.2. Very near the isoelectric point there is a minimum with a value about equal to that obtained at pH 6. On the acid side of the isoelectric point there is a second maximum followed by a second minimum. Interpreted in terms of the Kuhn-Guth or Eisenschitz theories, these data indicate that in the alkaline range there is a dissociation of the molecule into simple, less asymmetrical units, and that, as one

TABLE II

*Specific Viscosity of Tobacco Mosaic Virus Protein in Buffers at Various Hydrogen Ion Concentrations**

Buffer system	pH	$\frac{(\eta/\eta_0) - 1}{C}$
NaCl-NaOH.....	10.61	9.4
Na ₂ B ₄ O ₇ -HCl.....	8.92	29.2
“.....	8.27	35.2
“.....	7.69	43.0
NaAc-HCl.....	6.51	47.6
“.....	6.03	47.6
“.....	5.53	49.0
“.....	4.97	139.4
“.....	4.24	223.0
“.....	3.74	50.2
NaCl-HCl.....	3.03	95.8
“.....	2.12	49.8

* The concentration of protein is expressed as gm. per cc., ionic strength, $S = 0.02$.

approaches the isoelectric point from either side, one encounters, first, an end to end association of molecules to form new particles of greater asymmetry, and, finally, a side to side association of the long particles to form less asymmetrical crystals of length and thickness within the range of microscopic visibility in which the rod-shaped molecules are arranged side by side and end to end.

Studies of the double refraction of flow of the tobacco mosaic virus protein in buffers of various pH values confirm in general the conclusion drawn from the viscosity data. As is seen in Fig. 1, the stream double refraction of tobacco mosaic virus protein,

measured by the method described by Lauffer and Stanley (14), is at a maximum in the region of the isoelectric point and decreases above pH 6 and below pH 3. The increased double refraction in the region of the isoelectric point may be regarded as being due to the end to end association of the rod-shaped molecules to form particles of greater length, which, therefore, orient to a greater extent. It should be noted that stream double refraction begins to increase at a pH value somewhat higher than that at which the viscosity begins to increase, and that no minimum is observed very near the isoelectric point. The difference in behavior be-

EFFECT OF pH ON S. D. R. OF TOBACCO MOSAIC VIRUS PROTEIN

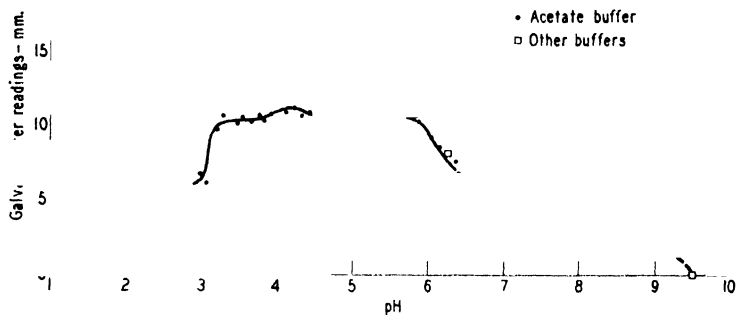


FIG. 1. The effect of pH on the stream double refraction of tobacco mosaic virus protein. The measurements were made on solutions containing about 0.25 mg. of tobacco mosaic virus protein per cc. Double refraction is reported as galvanometer deflections in mm., to which it is approximately proportional.

tween stream double refraction and viscosity very near the isoelectric point is probably due to the fact that the viscosity is a function of the length and thickness of the particles, whereas stream double refraction is concerned only with the length. If very thin long particles associate side to side at the isoelectric point, the viscosity should decrease, but the stream double refraction may well be unaffected. These stream double refraction data are entirely consistent with the observation of Mehl that the rotational diffusion constant of the protein, as estimated from stream double refraction data, is much less around pH 4.5 than at 6.8 (15). This rotational diffusion constant is regarded as being

inversely proportional to the cube of the particle length, and hence Mehl's results indicate particles of much greater length at pH 4.5 than at pH 6.8.

Viscosity As Function of Ionic Strength—In Table III are presented the results of a study of the effect of electrolytes on the specific viscosity of tobacco mosaic virus protein. It may be seen that the specific viscosity decreases with increasing ionic strength of the electrolyte. It seems reasonable to suppose that this change is due to the electroviscous effect (Equation 2) (19). The electrokinetic potential, ζ , is regarded as being a function of the electrical charge on the colloidal particle and the thickness of the double layer; *i.e.*, the distance between the charged surface and the electrical center of the atmosphere of oppositely charged ions in the liquid surrounding the particle. This distance turns out

TABLE III
*Effect of Electrolytes on Specific Viscosity of Tobacco Mosaic Virus Protein**

Solvent	Ionic strength	pH	$\frac{(\eta/\eta_0) - 1}{C}$
Distilled water	Very small	6.5	57.87
NaAc-HCl	0.020	6.5	47.6
K ₂ HPO ₄ -KH ₂ PO ₄ (2.2:1)	0.053	7.3	46.0
“ (2.2:1)	0.119	7.1	39.6
“ (2.2:1)	0.238	7.2	42.7

* The concentration of protein is expressed as gm. per cc.

to be inversely proportional to the square root of the ionic strength. Hence, the electrokinetic potential, and therefore the electroviscous effect, would be expected to vanish in solutions of high ionic strength. In making a calculation of the asymmetry of the particle, it perhaps would seem to be well to use the minimum value for specific viscosity obtained in solvents of high ionic strength, because in such solvents both R and ζ found in Equation 2 are low and, hence, the specific viscosity of the protein would be due largely to its shape. Such a procedure would result in a lower value of the asymmetry than that reported for the protein. However, since this low value would probably be too low because of the orientation of the particles due to streaming in the viscometer, it seems desirable to allow these two errors to compensate each other partially.

SUMMARY

From measurements of the specific viscosity of tobacco mosaic virus protein solutions used in conjunction with sedimentation data, the size and shape of the tobacco mosaic virus protein molecule have been estimated. Two alternate sets of values are obtained, one corresponding to rod-like particles having a molecular weight of 42.6×10^6 , a diameter of $12.3 \text{ m}\mu$, and a length of $430 \text{ m}\mu$, and the other corresponding to rod-like particles having a molecular weight of 63.2×10^6 , a diameter of $11.5 \text{ m}\mu$, and a length of $725 \text{ m}\mu$. Both of these sets of values are of the same order of magnitude as the values obtained from stream double refraction, diffusion, ultrafiltration, and x-ray diffraction data. In terms of a model arbitrarily chosen to have the dimensions of the first set of values, it was shown that a second component, whose particles are formed by the end to end association of two rod-like molecules resembling the model, should have a sedimentation constant of 202×10^{-13} as compared with 174×10^{-13} for the original component. Preparations of tobacco mosaic virus protein showing double boundaries in the ultracentrifuge have components with sedimentation constants of 174×10^{-13} and 200×10^{-13} .

The variations of viscosity and double refraction of flow of the protein with changes in hydrogen ion concentration are discussed. Both of these characteristics increase in the region of the isoelectric point, but only the viscosity falls sharply to a minimum very near the isoelectric point. This behavior is regarded as being due to the end to end association of rod-like molecules, followed by the side to side association of the long rods as one approaches the isoelectric point from either side. The viscosity was found to decrease upon the addition of electrolytes, an effect probably due to the electrokinetic potential of the particles.

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PROPERTIES OF THE LATENT MOSAIC VIRUS PROTEIN

BY HUBERT S. LORING

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton)

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The isolation and characterization by means of ultracentrifugal procedures of a heavy material that possessed the properties of the latent mosaic virus (*X* virus) have been described (1, 2). This material was considered to be a protein, because it gave the usual tests for a protein and, in the analytical ultracentrifuge, proved to be relatively homogeneous. It was prepared by high speed centrifugation of the clarified infectious juice and was obtained in yields of about 0.1 mg. per cc. of juice centrifuged. Since a solution of 0.1 mg. per cc. of the purified material produced about as many lesions as the original juice, it was considered to be about 10,000 times as infectious.

Recently, Bawden and Pirie obtained the latent mosaic protein by repeated precipitation with ammonium sulfate and treatment with pancreatin (3). Various properties, including the ability of a 2 per cent solution to form a birefringent lower layer, were reported. Bawden and Pirie, however, considered their preparations to be irreversibly aggregated, because, unlike the clarified infectious juice, the purified virus failed to pass filters of 400 m μ average pore diameter.

In order to obtain sufficient quantities of the protein for chemical studies, the centrifugation of large volumes of juice would be necessary. In the present work, attempts were therefore made to bring about a preliminary concentration of the protein without changing its specific activity. Several methods were used and the effect on the yield and specific activity of the protein was determined. Virus protein of high specific activity was prepared and analyzed, and a number of its physical and chemical properties were determined. Ultrafiltration experiments were carried out to

determine whether high speed centrifugation caused an irreversible aggregation with loss of filtrability through ultrafilters, and a decrease in specific activity. This paper presents the results of these investigations.

EXPERIMENTAL

Both *Nicotiana glutinosa*, L., and Turkish tobacco plants have been used as a source of virus. The plants were inoculated and harvested and the juice was extracted as previously described (2). In some cases K_2HPO_4 was added to the pulp and in others this treatment was omitted. The juice was clarified by filtration with suction through diatomaceous earth (an equal mixture of Standard and Hyflo supercel). Activity measurements on the juice before and after filtration showed that the amount of virus lost by this procedure was comparable to that lost by gravity filtration through paper. The virus protein was isolated by the ultracentrifugation of the clarified juice directly or by the ultracentrifugation of concentrates prepared after precipitation of the virus protein either with HCl at pH 4, with an equal volume of alcohol at pH 5, with 20 and 30 per cent $(NH_4)_2SO_4$, or with 30 per cent potassium citrate. The precipitated protein was in each case removed by filtration on Standard supercel and redissolved in about one-twentieth the original volume of 0.01 M buffer or water at pH 7 to 8. The virus protein was then separated from the normal protein and other plant constituents by three sedimentations in a field of about 50,000 times gravity. The pellets obtained after each sedimentation were redissolved in 0.01 M buffer or water and the solutions were filtered through a thin layer of Standard supercel and again ultracentrifuged. All operations were carried out at about 5°. The protein concentrations of the final solutions were determined by Kjeldahl analysis.

The yields of virus protein obtained in experiments in which different methods of concentration were applied to portions of the same juice are shown in Table I. The yields for virus sedimented three times have varied from about 1 to 9 mg. per 100 cc. of juice. In most cases the yields were about 30 per cent higher when the juice was sedimented directly than those obtained after either ammonium sulfate or potassium citrate precipitation. After precipitation at pH 4 with HCl and at pH 5 with alcohol, only

about 20 per cent as much virus was obtained as by the ultracentrifugation of juice. In no case was a yield greater than about 10 mg. per 100 cc. obtained. Activity tests on the original juice

TABLE I
Comparative Yields of Virus Protein Obtained by Ultracentrifugation of Clarified Juice and Protein Concentrates

Sample No.*	Method of extraction	Type of treatment†	Volume of juice	Times sedimented	Yield
			cc.		mg. per 100 cc.
G5	Without K_2HPO_4	Ultracentrifugation of juice	220	3	7.0
		30% $(NH_4)_2SO_4$ and ultracentrifugation	230	3	5.1
		30% $K_3C_6H_5O_7$ " "	240	3	6.3
G6	" "	Ultracentrifugation of juice	500	3	6.2
		20% $(NH_4)_2SO_4$ and ultracentrifugation	500	3	4.6
		30% $K_3C_6H_5O_7$ " "	1500	3	4.3
		HCl at pH 4 " "	500	3	1.4
		Alcohol at pH 5 " "	500	3	1.0
G9	" "	Ultracentrifugation of juice	1240	3	9.3
G3	" "	" " "	224	2	6.5
		20% $K_3C_6H_5O_7$ and ultracentrifugation	400	2	4.0
G2	4% K_2HPO_4	Ultracentrifugation of juice	250	3	6.2
		15% $(NH_4)_2SO_4$, 6% K_2HPO_4 , and ultracentrifugation	70	3	4.0
T6	4% "	Ultracentrifugation of juice	400	2	2.8
		15% $(NH_4)_2SO_4$, 6% K_2HPO_4 , and ultracentrifugation	500	3	2.2

* The samples labeled G were obtained from *Nicotiana glutinosa* plants; that labeled T from Turkish tobacco plants.

† When prepared by the ultracentrifugation of juice, the sedimented virus was redissolved in water or 0.01 M phosphate buffer at pH 7 to 8. When precipitated by chemical treatment, the precipitated protein was redissolved in water or 0.01 M phosphate and the resulting solution was ultracentrifuged.

and on the supernatant liquid after sedimentation of the virus have shown that about 99 per cent of the virus was sedimented, and, as the subsequent treatments likewise caused small losses of

virus, it seems likely that the maximum yields reported represent about 60 to 80 per cent of the virus present in the juice.

Care was taken during the first centrifugation of the protein concentrates to keep the temperature near 0°. If the temperature was allowed to approach 15-20°, the amount of darkly pigmented materials sedimented along with the virus protein was greatly increased and could later be removed only with considerable difficulty. The formation of the insoluble pigment appeared to proceed most rapidly in the $(\text{NH}_4)_2\text{SO}_4$ solutions. If the solutions were kept cold, the purified virus protein was readily obtained after three sedimentations as transparent birefringent pellets which formed slightly opalescent solutions in distilled water and contained only traces of the salt used for preliminary precipitation. In the analytical ultracentrifuge, these samples were free from detectable amounts of ultraviolet-absorbing impurities of low molecular weight.¹ The supernatant liquids from the third centrifugation were almost entirely free from protein impurities of low molecular weight that could be precipitated with hot trichloroacetic acid. As the sedimentation rate of the virus protein is decreased in more concentrated solutions, the length of time necessary for complete sedimentation depends on the concentration. Solutions that contain more than 2 or 3 mg. of virus protein per cc. require 1½ to 2 hours in a field of 50,000 times gravity for about 99 per cent sedimentation.

Specific Activities of Virus Protein—As the number of lesions obtained after inoculation with latent mosaic virus, like tobacco mosaic virus, varies over a wide range, depending on the susceptibility of the test plants, it is difficult to determine the specific activity of a particular sample from the number of lesions obtained in a single test. The relative activities of two samples may be determined, however, by comparing the number of lesions produced by equal concentrations of each on half leaves of the same plants. Similarly, the activities of several samples may be determined by comparing each on half leaves against a common control. This method, in the case of tobacco mosaic virus, has been found sufficiently sensitive for the detection of differences in concentration of 10 to 20 per cent (4) and was, therefore, used in the present experiments.

¹ Wyckoff, R. W. G., unpublished work.

The relative activities of different samples prepared by the ultracentrifugation of juice from both Turkish tobacco and *Nicotiana glutinosa* plants are shown in Table II. It may be seen that samples isolated by this procedure have, in general, shown the same activity, provided they were isolated within a few days of

TABLE II

Relative Activities of Latent Mosaic Virus Protein Prepared by Ultracentrifugation of Juice from Turkish Tobacco and Nicotiana glutinosa Plants

Host plant	Method of extraction	Date isolated	Date of test	Relative activities*			
				Test 1	†	Test 2	t
Turkish	3% Na_2HPO_4	Dec. 15, 1936	Mar. 18, 1937	500	0.5		
"	4% K_2HPO_4	Feb. 26, 1937		525			
<i>N. glutinosa</i>	4% "	" 19, 1937	Feb. 27, 1937	339	0.8		
Turkish	4% "	" 26, 1937		316			
<i>N. glutinosa</i>	4% "	" 19, 1937	June 31, 1937	492	7.6	314	
" "	Without phosphate	May 13, 1937		1263		655	
" "	" "	Oct. 13, 1937	May 25, 1938	939	3.4	772	
" "	" "	May 20, 1938		1206		1066	
" "	" "	July 27, 1938	Aug. 10, 1938	553	1.4	1070	
" "	" "	" 27, 1938		631		1045	
" "	" "	" 27, 1938	Aug. 10, 1938	114	1.0	156	
" "	" "	" 29, 1938		96		148	

* Number of lesions produced on sixteen to twenty-four half leaves of young Turkish tobacco plants at a concentration of 10^{-5} gm. of protein per cc. in 0.01 M phosphate buffer at pH 7.

† The ratio of the mean difference to the standard error of the mean difference. Values of 2.1 or greater indicate odds of at least 20:1 for significance.

each other. Those that were several weeks old, although stored in a refrigerator, have in most cases produced fewer lesions per unit weight of native protein than freshly prepared samples. Virus protein prepared by this method was used, therefore, as a standard in the tests in which the relative activity of samples

obtained after chemical treatment was determined. The results of the activity tests on samples isolated from the same juice by the different procedures described above are shown in Table III. These results show an appreciable variation in relative or specific

TABLE III

Relative Activities of Samples of Latent Mosaic Virus Protein Prepared from Same Juice by Different Treatments

Preparation No.	Type of treatment*	Relative activities†			
		Test 1	t‡	Test 2	t
G5	Ultracentrifugation of juice 30% (NH ₄) ₂ SO ₄ + ultracentrifugation	471		465	
		283	7.3	367	3.0
	Ultracentrifugation of juice 30% K ₃ C ₆ H ₅ O ₇ + ultracentrifugation	479		255	
		309	4.0	236	1.0
G6	Ultracentrifugation of juice 20% (NH ₄) ₂ SO ₄ + ultracentrifugation	556		601	
		610	1.5	551	1.0
	Ultracentrifugation of juice 30% K ₃ C ₆ H ₅ O ₇ + ultracentrifugation	729		743	
		688	1.0	770	0.5
	Ultracentrifugation of juice HCl at pH 4	676		922	
		509	2.6	658	4.8

* When prepared by the ultracentrifugation of juice, the sedimented virus was redissolved in water or 0.01 M phosphate buffer at pH 7 to 8. When precipitated by chemical treatment, the precipitated protein was redissolved in water or 0.01 M phosphate and the resulting solution was ultracentrifuged.

† Number of lesions produced at a concentration of 10⁻⁵ gm. of protein per cc. in 0.01 M phosphate buffer on twenty-four half leaves of young Turkish tobacco plants.

‡ The ratio of the mean difference to the standard error of the mean difference. Values of 2.1 or greater indicate odds of at least 20:1 for significance.

activity, depending on the type of treatment to which the sample had been exposed. Preliminary concentration with either 20 per cent ammonium sulfate or 30 per cent potassium citrate gave samples with about the same specific activity as the control obtained by sedimentation alone, but precipitation with 30 per

cent ammonium sulfate or with HCl at pH 4 resulted in preparations of lower specific activities.

It seemed likely that the low activity described above could have resulted because of the exposure to strong electrolyte. This question was further studied by adding $(\text{NH}_4)_2\text{SO}_4$ to samples of virus protein prepared by the ultracentrifugation of juice. In one experiment the virus was reprecipitated from its aqueous solution five times, and in another it was allowed to stand in 10 per cent $(\text{NH}_4)_2\text{SO}_4$ for a day at room temperature. In each case the solution was freed from salt by dialysis at 4° , and the relative activity of the virus was compared with a control solution which received the same treatment without the addition of $(\text{NH}_4)_2\text{SO}_4$. The activity of virus protein that had been prepared by repeated precipitation with $(\text{NH}_4)_2\text{SO}_4$ and treatment with pancreatin, as used by Bawden and Pirie (3), was also determined. One such sample, which was obtained after five precipitations, one treatment with pancreatin, and one ultracentrifugation, and gave a liquid crystalline bottom layer, was compared with two samples that had been prepared by two sedimentations from juice as described above. The activity data for the three experiments are summarized in Table IV. It may be seen that, on a protein basis, the control samples in every case were several times as active as those that had been treated with $(\text{NH}_4)_2\text{SO}_4$. As the number of lesions produced by the sample prepared by the method of Bawden and Pirie was about the same at a 10^{-4} gm. per cc. concentration as that produced by a 10^{-5} gm. per cc. concentration of the sample prepared by ultracentrifugation alone, its specific activity was of the order of one-tenth that of the latter. These results are in agreement with those in which one precipitation with 30 per cent ammonium sulfate appeared to cause a loss of activity. Bawden and Pirie did not compare the relative activities of their samples, but from the variation in the number of lesions obtained on dilution it seems likely that their preparations differed greatly in relative activity. Because appreciable loss of virus activity resulted after precipitation by 30 per cent ammonium sulfate and contact with 10 per cent ammonium sulfate at room temperature, it is probable that one precipitation with 20 per cent ammonium sulfate or 30 per cent potassium citrate at 4° also caused a loss of virus activity which was not detected because of the low sensitivity

of the infectivity measurements. These methods are useful in preparing considerable amounts of virus, but high speed centrifugation carried out at low temperatures, in the absence of strong electrolytes, appears to provide the most satisfactory method for

TABLE IV

Relative Activities of Virus Protein Subjected to Treatment with $(\text{NH}_4)_2\text{SO}_4$

Type of treatment	Relative activities*					
	Concen- tration	Test 1	t †	Concen- tration	Test 2	t
	gm. protein per cc.			gm. protein per cc.		
Control‡	10^{-5}	892				
Precipitated 5 times with $(\text{NH}_4)_2\text{SO}_4$	10^{-5}	390	8.6			
Control‡	2×10^{-5}	777		2×10^{-5}	646	
24 hrs. contact with 10% $(\text{NH}_4)_2\text{SO}_4$ at room tem- perature	2×10^{-5}	291	5.8	2×10^{-5}	300	4.7
Twice sedimented virus, Sample 1	10^{-5}	808		10^{-5}	416	
Prepared by method of Bawden and Pirie (3)	10^{-5}	73	8.8	10^{-4}	494	1.5
Twice sedimented virus, Sample 2	10^{-5}	553		10^{-5}	487	
Prepared by method of Bawden and Pirie (3)	10^{-5}	102	10.3	10^{-5}	83	6.1

* Number of lesions produced on twenty-four half leaves of young Turkish tobacco plants.

† The ratio of the mean difference to the standard error of the mean difference. Values of 2.1 or greater indicate odds of at least 20:1 for significance.

‡ The controls in these experiments consisted of virus protein samples that had received the same treatment in the absence of ammonium sulfate.

the preparation of latent mosaic virus protein of the highest and most constant specific activity.

General Properties—Latent mosaic virus protein in water, like tobacco mosaic virus protein, gives opalescent solutions which show a Tyndall cone. The amount of opalescence depends on the concentration and the amount of treatment to which the sample has been subjected. Freshly prepared solutions show a very

slight opalescence, but after standing at room temperature or in the presence of 10 per cent $(\text{NH}_4)_2\text{SO}_4$ for a day become more opaque. In a similar way, aqueous solutions that are clear become more opalescent if adjusted to about pH 4.0, and the protein in this case may be sedimented by means of the ordinary laboratory centrifuge. Unlike tobacco mosaic, however, samples of latent mosaic do not form crystals when brought to the isoelectric point, although a slight sheen is observed.

The latent mosaic virus protein is much less stable than that of tobacco mosaic. Solutions in 0.1 M or 0.01 M phosphate at pH 7 showed an immediate drop in activity after standing for 24 hours at room temperature. After longer periods than 24 hours, some bacterial decomposition usually became noticeable at pH 7, but virus activity was retained even after 10 days. At pH 9 no bacterial putrefaction occurred. One solution held at this reaction at room temperature remained infectious for over 4 months, although there was a gradual loss of activity, as compared with the control kept at 4°. A solution of tobacco mosaic virus protein that had stood at room temperature for about a month lost only 20 per cent of its virus activity, as compared with a control kept at 4°. The latent mosaic virus protein was denatured and virus activity was lost when a 0.1 per cent solution in 0.1 M phosphate buffer at pH 7 was heated to 64° for 5 minutes. Virus activity was also lost when the purified virus protein in distilled water was dried *in vacuo* while frozen.

Analysis of Purified Virus Preparations—Chemical analyses of several different samples for C, H, N, S, P, and carbohydrates are shown in Table V. The analyses are those that would be expected for a nucleoprotein containing about 6 per cent nucleic acid and are in essential agreement with those published by Bawden and Pirie. The protein gave the qualitative tests for guanine and pentose, and a material with the qualitative solubility of yeast nucleic acid was obtained by treatment with several volumes of glacial acetic acid (3, 7). However, the ratio of carbohydrate to phosphorus in the protein, as shown in Table V, was almost twice that found for yeast nucleic acid. If the virus nucleic acid be assumed to give the same carbohydrate to phosphorus ratio as yeast nucleic acid, these results show that other carbohydrates or compounds which react with orcinol and sulfuric acid must also

be present in the virus protein. The ratio of carbohydrate to phosphorus for ultracentrifuged samples is somewhat higher than that of about 6:1 obtained by Bawden and Pirie for their preparations, but even in the latter case is still about 50 per cent too high for all the carbohydrate and phosphorus to be combined as nucleic

TABLE V
Analyses of Latent Mosaic Virus Protein

Sample*	C	H	N (Dumas)	S	P		Carbohydrate† as glucose‡	N:P	Carbohydrate:P
					Pregl	King (5)†			
	per cent	per cent	per cent	per cent	per cent	per cent	per cent		
Latent mosaic, No. 1	47.8	7.27	14.58						
" " " 2	47.7	7.6	14.54						
" " " 3					0.69				
" " " 4						0.63	4.3	25:1	6.8:1
" " " 5						0.56	4.0	28:1	7.1:1
" " " 6						0.53	4.2	30:1	7.9:1
" " " 7						0.59	3.9	27:1	6.6:1
" " " 8	49.1	6.84	15.8-16.1	1.1	0.52			31:1	
Yeast nucleic acid						7.2	27		3.8:1
						7.9	28		3.5:1

* Samples 1, 2, 4, and 8 were prepared by ultracentrifugation of the clarified juice. Samples 3 and 6 were precipitated once at 5° with 30 per cent potassium citrate and then ultracentrifuged. Samples 5 and 7 were precipitated once at 5° with 20 per cent ammonium sulfate and then ultracentrifuged. Samples 1 and 2 were dialyzed at pH 8 for 5 days, precipitated with acetone, and dried to constant weight over P₂O₅. Samples 3 and 8 were dried *in vacuo*, while frozen, and then to constant weight over P₂O₅. Samples 4 to 7 consisted of aqueous solutions of active virus.

† The values in these columns were calculated from analyses for nitrogen, phosphorus, and carbohydrate, and are based on the assumption that the nitrogen content of these samples was 15.8 per cent.

‡ Carbohydrate was determined with orcinol and sulfuric acid (6).

acid. The purified virus, therefore, is probably not simply nucleoprotein in nature. It is unlikely that the higher carbohydrate content of the ultracentrifuged samples was due to the sedimentation of dark pigments rich in carbohydrates, for, when the juice from young succulent plants is used and the temperature is kept

at about 5°, pellets almost entirely free from pigment are obtained. The values for carbohydrates shown in Table V were obtained with such samples which were almost completely colorless, and in most cases gave liquid crystalline solutions.

The nitrogen values found for the ultracentrifuged samples were slightly lower and the phosphorus values slightly higher than those reported by the authors mentioned above. The differences are well brought out if the N:P ratios are compared. Samples prepared by the ultracentrifugation of juice gave ratios of from 25 to 31:1 as compared with ratios in the range from 31 to 43:1 for those obtained by Bawden and Pirie.

Isoelectric Point of Latent Mosaic—It has been shown (3, 8) that the latent mosaic virus is precipitated at or below pH 4. Such experiments indicate that the latent mosaic virus protein may have an isoelectric point near pH 4. Cataphoresis experiments with collodion particles coated with latent mosaic virus protein showed that the isoelectric point as determined in 0.01 M phthalate-phosphate-borate buffer varied with the previous treatment to which the sample had been subjected. The procedure used in the cataphoresis experiments was as follows: 2 drops of a 1 per cent collodion suspension were added to 1 cc. of a solution containing 1 mg. of virus protein per cc. and the suspension was allowed to stand for several minutes. It was then diluted with 400 parts of 0.01 M phthalate-phosphate-borate buffer, and examined in the cataphoresis cell. Freshly prepared samples obtained after high speed centrifugation of infectious juice have shown an isoelectric point of about 3.7. Samples obtained after preliminary precipitation with $(\text{NH}_4)_2\text{SO}_4$ or potassium citrate and high speed centrifugation have proved to be isoelectric at about pH 4.4, while other samples prepared by extraction with 3 per cent dibasic phosphate at about pH 7 and high speed centrifugation have given isoelectric points of about pH 5. It seems likely that such changes in cataphoretic behavior have been brought about by contact of the protein with electrolytes.

pH Stability Range—In considering the effect of pH on virus activity, it is necessary to determine whether the failure to obtain infection is due to an effect on the host or to an inactivation of the virus. The effect would be observed immediately in the first case, whereas in the second the amount of virus activity would be ex-

pected to depend on the length of time the virus had been exposed to a particular hydrogen ion concentration. Virus solutions adjusted to various pH values between 2.5 and 11 were tested for activity immediately and after they had stood at 0° for 12 hours, and solutions which had stood at the various pH levels for 12 hours were tested for activity after they had been readjusted to pH 7.5. In this way it could be determined whether there was an immediate or gradual loss of virus activity at some pH and whether the loss in activity could be regained after readjustment to pH 7.5. In the experiments in which the samples were tested for activity at the different hydrogen ion concentrations, solutions at 0° containing 10^{-5} gm. of protein per cc. in 0.1 M phthalate-phosphate-borate were prepared and their activities were determined from the relative number of lesions produced by each on about twenty-four half leaves compared to a control solution of the same concentration at pH 7.5. In experiments in which the hydrogen ion concentrations were readjusted before the activity measurements were made, solutions in the same buffer and over the same pH range containing 10^{-4} gm. of virus protein were prepared and diluted to 10^{-6} gm. per cc. with 0.1 M phosphate buffer at pH 7.5 and 0° for the infectivity measurements. The control solution was prepared in the same way by dilution of a sample in the phthalate-phosphate-borate buffer at pH 7.5 with 0.1 M phosphate. In this way the solutions at different pH values were readjusted to within about 0.5 pH unit of the control solution at pH 7.5 and in each case contained very nearly the same total salt concentration.

The results of the activity measurements made at the different pH values and after adjustment to pH 7.5 are shown in Fig. 1 (Curves A, B, and D). The relative activity of each solution is expressed as per cent of the control solution at pH 7.5. The data have been summarized in Table VI. The results show that about the same number of lesions were obtained over the pH range from 6 to 9, whether the solutions were tested immediately or after 12 hours at 0°. When tested immediately, solutions at pH 5 and 10 also produced the same number of lesions, but after standing for 12 hours they both showed a loss of about 40 per cent of their original activity. Below pH 5 and above pH 10 significantly fewer lesions were obtained when tested both immediately and

after 12 hours. When solutions which had stood between pH 4 and 5 for 12 hours were readjusted to pH 7.5, however, as shown in Fig. 1 (Curve D), they produced as many lesions as their controls. The loss of activity which took place on standing between pH 4 and 5 was, therefore, reversible, and, as the protein is iso-electric over this range, no doubt represented aggregation of virus particles. Below pH 4 and at pH 10 or higher the inactivation was not found to be reversible. At pH 3 and 11 some virus activity was found if the solutions were tested immediately, although it was of the order of 1 per cent or less than the control solution. After 12 hours these solutions failed to show any virus

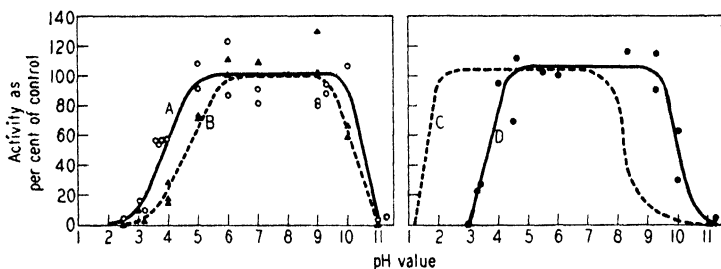


FIG. 1. Activity-pH curves of latent mosaic and tobacco mosaic virus. Curve A, latent mosaic virus, tested immediately at various pH values; Curve B, latent mosaic virus, tested after 12 hours at various pH values; Curve C, tobacco mosaic virus, data from Best and Samuel (9, 10), tested after 12 hours at various pH values and readjustment to pH 7; Curve D, latent mosaic virus, tested after 12 hours at various pH values and readjustment to pH 7.5.

activity. The pH inactivation of tobacco mosaic virus as found by Best and Samuel (9, 10) is also reproduced in Fig. 1 (Curve C). Of striking interest is the greater stability of the latent mosaic virus over the range from pH 8.5 to 10. At these pH values tobacco mosaic is largely inactivated. Sedimentation studies with latent mosaic virus at various pH values have been made by Wyckoff.¹ They show that at pH values where virus activity was lost molecular homogeneity was also destroyed.

The differences in lesion counts were not tested for their statistical significance in the above experiments. In similar experiments, however, in which the standard error has been calculated,

TABLE VI

Effect of Different Hydrogen Ion Concentrations on Activity of Latent Mosaic Virus

pH	Tested immediately after exposure			Tested 12 hrs. after exposure					
	No. of lesions on 24-28 half leaves		Virus remaining active*	No. of lesions on 24-28 half leaves		Virus remaining active*	No. of lesions on 24-28 half leaves		Virus remaining active*
	At various pH	Control at pH 7.5		At various pH	Control at pH 7.5		After readjustment to pH 7.5	Control at pH 7.5	
			per cent			per cent			per cent
2.5	18	430	4	0	1059	0			
3.0	40	259	16	30	270	11	11	737	2
3.2	44	443	10	12	794	2	118	538	22
3.4							70	263	27
3.6	812	1429	57						
3.7	679	1253	54						
3.8	592	1059	56	94	598	16			
4.0	281	498	57	146	505	29	509	541	94
				80	430	19			
4.5							701	1013	69
4.6							611	549	111
5.0	214	236	91	213	292	73			
	1029	953	108	387	537	72			
5.5							351	344	102
6.0	284	330	86	337	306	111	1028	1026	100
	1097	899	122	399	399	100			
7.0	1356	1512	90	691	631	109			
	702	857	82						
8.3							690	593	116
9.0	872	1061	82	806	618	130			
	499	629	80	776	759	102			
9.3	457	551	83				543	474	115
	523	588	89				805	895	90
10	387	365	106	449	683	66	481	757	64
				379	645	59	183	494	37
11.0	10	350	3	0	700	0			
11.3	29	654	5				29	654	5

* Percentage number of lesions obtained with test solutions as compared with controls. Values within 15 per cent of the controls are usually not significantly different.

differences of 20 per cent or more in the number of lesions obtained on half leaves usually gave odds for significance of at least 20:1. In cases in which the test samples caused only about 30 per cent as many lesions as the control, it is likely that the virus concentration was of the order of only 10 per cent that of the control. In those cases in which fewer than 10 per cent as many lesions were obtained as with the control, it is likely that the test solution contained only about 1 per cent of the active virus present in the control. In the latter cases the activities were also determined with whole leaves. The results were essentially the same as those shown in Table VI.

Serological Properties—The presence of a specific antigen in the juice from plants infected with the latent mosaic virus has been shown by several investigators (11–13). That the protein isolated by the above procedure is this antigen is shown by the fact that antiserum to the protein gives a precipitate with infectious juice but fails to react with the juice from normal plants. The protein reacts with its homologous antisera under the conditions used by Bawden and Pirie to give a just detectable precipitate at a protein dilution of $1:10^7$. Bawden and Pirie have reported titers of $1/(6 \times 10^6)$ for their preparations of latent mosaic. It is unlikely that these differences are significant.

Ultrafiltration Experiments—It seemed desirable to determine first the effect of ultracentrifugation alone on the ability of the resuspended virus to pass collodion membranes² of about 250 m μ average pore diameter. Membranes of this pore size were chosen because it was found in experiments with clarified infectious juice that such a pore size represented nearly the minimum which would allow the passage of most of the virus at a 1:5 dilution in the presence of nutrient broth and 0.1 M phosphate at pH 8.5. A sample of infectious juice, clarified by filtration through celite, was centrifuged under the conditions used for isolation of the virus, and the sedimented virus was resuspended in the supernatant juice. In some instances the sedimented pellet was redissolved immediately by mechanical mixing and in others it was allowed to stand overnight at 4° before the supernatant juice was stirred. A portion of the suspension was then diluted with nu-

² The author wishes to express his thanks to Dr. J. H. Bauer and Dr. T. P. Hughes for some of the membranes used in these experiments.

trient broth, 1 M K_2HPO_4 , water, and NaOH, so the final solution consisted of juice and nutrient broth at approximately a 1:5 dilution in 0.1 M phosphate at pH 8.5. The precipitated inactive protein was centrifuged and the filtration was carried out as outlined by Thornberry (14). The amount of virus in the filtrate was then determined by comparing the number of lesions produced on half leaves by the filtrate and the original solution. As a

TABLE VII
Effect of Sedimentation on Ultrafiltration of Latent Mosaic Virus

Experiment No.*	Virus solution†	No. of lesions on 24 half leaves		Virus in filtrate
		Before	After	
1	Infectious juice	737	642	87
	Sedimented virus resuspended in supernatant juice immediately	746	539	72
2	Infectious juice after 24 hrs. at 4°	371	303	82
	Sedimented virus resuspended in supernatant juice after 24 hrs. at 4°	229	90	39
3	Infectious juice	596	80	13
	Sedimented virus resuspended in supernatant juice immediately	708	78	11
	Infectious juice after 24 hrs. at 4°	1497	260	17
	Sedimented virus resuspended in supernatant juice after 24 hrs. at 4°	1025	35	3.4

* In Experiments 1 and 2, membranes of 250 μ average pore diameter were used; in Experiment 3, membranes of 200 μ average pore diameter were used.

† All samples were diluted before filtration with nutrient broth, 1 M K_2HPO_4 , NaOH, and water to give solutions containing juice and broth at a 1:5 dilution in 0.1 M phosphate at pH 8.5.

control, a sample of the original juice after dilution with broth, etc., was filtered at the same time through a membrane of the same size, and the relative activities of the filtrate and original solution were also compared. The experiments recorded in Table VII show no striking differences between the ability of the sedimented and the untreated virus to pass filters of 200 to 250 μ average pore diameter. In those instances in which the pellet had not been immediately resuspended, the virus appeared to

filter somewhat less readily than the control sample of juice, but the degree of difference was small and possibly was not significant. From these experiments the conclusion appears to be justified that

TABLE VIII
Ultrafiltration of Purified Latent Mosaic Virus

Ex- peri- ment No.	Times virus sample was sedimented	Concen- tration	Potassium phosphate buffer*			No. of lesions on 24 half leaves		Virus in fil- trate
						Before	After	
		<i>gm. per cc.</i>	<i>M</i>	<i>pH</i>	<i>mμ</i>			<i>per cent</i>
1†	2	10 ⁻⁴	0.01	6.7	250	About 100 per leaf	No infec- tion	0
	2	10 ⁻⁴	0.01	8.5	250	" "	5 plants of 8 posi- tive	<1
2	2	4 × 10 ⁻⁵	0.1	8.5	250	1000	68	7
3	3	2 × 10 ⁻⁵	0.1	8.5	300	667	375	56
						813	626	77
4	2 (after so- lution in 10% (NH ₄) ₂ - SO ₄ for 24 hrs.)	4 × 10 ⁻⁵	0.1	8.5	300	658	209	32
5	Control	4 × 10 ⁻⁵	0.1	8.5	300	930	608	65
	2	2 × 10 ⁻⁵	0.1	8.5	450	354	465	130
	2	2 × 10 ⁻⁵	0.1	8.5	450	413	379	92
	2	2 × 10 ⁻⁵	0.1	8.5	250	314	42	13

* The solvent in each case contained broth at a 1:5 dilution. In Experiment 5, virus-free supernatant juice at a 1:5 dilution was also used. It consisted of the upper 30 per cent of the supernatant liquid obtained after the ultracentrifugation of the infectious juice.

† In this experiment the original solutions and filtrates were tested on whole leaves of different groups of plants.

sedimentation alone, in the case of latent mosaic virus, does not cause an appreciable aggregation which is irreversible at pH 8.5.

Filtration experiments were also carried out with virus protein that had been purified by two and three sedimentations, and after treatment with (NH₄)₂SO₄. These experiments, summarized in Table VIII, show a great difference in filtrability, depending on the

solvent and pH. In the presence of broth and 0.1 M phosphate at pH 8.5, virus protein purified by ultracentrifugation alone could be filtered through membranes of 450 m μ average pore diameter with no detectable loss of virus activity, while in distilled water or 0.1 M phosphate buffer at pH 7, the virus failed to pass either a 450 m μ average pore diameter collodion or a Seitz filter. In the presence of broth and phosphate, the purified virus also passed a membrane of 250 m μ average pore diameter, but in relatively small amount compared to once sedimented virus redissolved in juice. It appears, therefore, that purified virus protein obtained after several centrifugations had undergone some change, which could be detected by a membrane of 250 m μ average pore diameter and was not reversed under the conditions used for filtration. Filtration experiments with samples of purified protein that had been allowed to stand in contact with 10 per cent (NH₄)₂SO₄ at room temperature for 24 hours showed that this material also passed a 300 m μ filter when dissolved in broth and 0.1 M phosphate at pH 8.5, but probably in smaller amount than a control solution of purified virus which had stood at room temperature for the same length of time in the absence of (NH₄)₂SO₄. About 32 per cent as many lesions were obtained with the filtrate as with the original solution in the case of the sample treated with (NH₄)₂SO₄, whereas about 65 per cent as many lesions as compared with the original were obtained for the control sample.

These results showed that the ability of purified latent mosaic to pass ultrafilters was largely dependent on the conditions under which the filtration was carried out. These differences may be due in part to different states of aggregation, but it seems more likely that in those experiments in which water and dilute buffer were used the virus was adsorbed on the filter surface. In view of the effect of changes in pH on relative viscosity found for tobacco mosaic virus protein (15), it seems likely that the state of aggregation of latent mosaic virus particles also varies with pH. The experiments described above show that latent mosaic virus is not irreversibly aggregated in the sense that it will not pass a filter of 450 m μ average pore diameter either after sedimentation or after contact with 10 per cent (NH₄)₂SO₄. However, the purified virus protein does differ from infectious juice in its ability to pass filters of about 250 m μ average pore diameter. It appears, therefore.

that some type of change, possibly aggregation, which is not reversed in 0.1 M phosphate at pH 8.5, has taken place, and that the amount of aggregation may be increased after the virus has been in contact with $(\text{NH}_4)_2\text{SO}_4$.

Particle Size of Latent Mosaic Virus—Because latent mosaic virus protein shows stream double refraction, it appears that, like tobacco mosaic virus protein, it consists of asymmetrical and probably rod-shaped particles. Such particles are present in the clarified juice as well as in purified preparations, for samples of filtered juice show a detectable amount of stream double refraction. The amount is small and cannot be seen readily but is detected easily in the apparatus described by Lauffer and Stanley (16) and is of the same order as that of juice from tobacco mosaic-infected plants diluted with about 25 parts of normal juice. It is possible to obtain some idea of the relative dimensions of rod-shaped particles from viscosity measurements with Kuhn's equation (17) and from the relative viscosity to calculate the dissymmetry constant (18) and molecular weight (19). As shown by Lauffer (20), a dissymmetry constant of 2.52 and a molecular weight of 42.5×10^6 are obtained for tobacco mosaic virus protein by this method. These values are in good agreement with those calculated from viscosity measurements of Frampton and Neurath (21). It seems likely, therefore, that they more nearly represent the true dissymmetry and molecular weight of the tobacco mosaic virus protein than the earlier values. In a few preliminary experiments the relative viscosity of a 0.1 per cent solution of purified latent mosaic virus protein in distilled water at 25° was about 1.09 as compared with the value of 1.057 found by Lauffer for tobacco mosaic virus protein. The ratio of particle length to width calculated from Kuhn's equation was 43.9:1 as compared to 35:1 for tobacco mosaic virus protein, and the dissymmetry constant and molecular weight for a specific volume of 0.73 and a sedimentation constant of 113 were 2.78 and 26×10^6 , respectively.³ It is prob-

³ It seems likely that the heavier component with a sedimentation constant of 200×10^{-13} found in some solutions of tobacco mosaic virus by Wyckoff (22) is due to the formation of end-to-end bimolecular aggregates of particles with a sedimentation constant of 174×10^{-13} , for, as shown by Lauffer (23), the calculated sedimentation constant of such particles agrees well with that determined by means of the ultracentrifuge. The sedimentation constant of end-to-end, bimolecular aggregates formed from latent

able that the latter value rather than that of 9×10^6 which was previously suggested (2) represents more nearly the true molecular weight of the latent mosaic virus protein purified by ultracentrifugation. A cylindrical particle of 26×10^6 molecular weight, 0.73 specific volume, and a ratio of length to width of 43.9:1 would have a diameter of about $9.8 \text{ m}\mu$ and a length of about $433 \text{ m}\mu$.

Layering Phenomenon—Preparations of latent mosaic virus protein obtained after treatment with $(\text{NH}_4)_2\text{SO}_4$ have been shown to form liquid crystalline solutions (3). Virus protein obtained by the centrifugation of juice also shows this property. If the birefringent pellet obtained after two or three sedimentations is

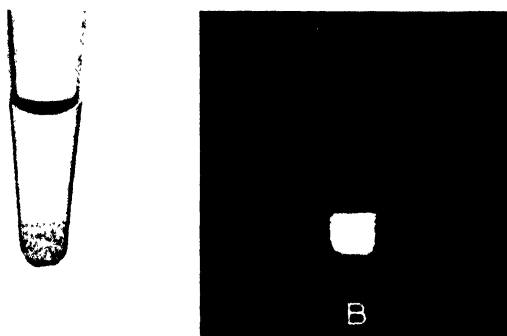


FIG. 2. Liquid crystalline bottom layer of latent mosaic virus protein: A, photographed between parallel polaroid plates; B, photographed between crossed polaroid plates. (Photographs by J. A. Carlile.)

dissolved in about 3 volumes of water, the resulting solution is usually liquid crystalline. If it is diluted to a protein concentration of about 1 per cent, the solution becomes opalescent and when examined between crossed polaroid plates is no longer birefringent. If such a solution is centrifuged at 3000 to 4000 R.P.M. for several hours, however, a liquid crystalline lower layer separates, as shown in Fig. 2.

mosaic virus particles having a sedimentation constant of 113×10^{-13} and the asymmetry described was calculated in a similar manner and found to be 131×10^{-13} . This value is the same as that found experimentally for the heavier component present in some preparations of purified latent mosaic virus.

While the more active virus protein obtained by high speed centrifugation shows the ability to form liquid crystalline layers, this property is not necessary for virus activity. Tobacco mosaic virus protein of low relative activity obtained after repeated precipitation with $(\text{NH}_4)_2\text{SO}_4$ and completely inactive preparations obtained after treatment with formaldehyde or H_2O_2 are known to give typical needle crystals. Such preparations also give liquid crystalline solutions, which are identical in appearance with those obtained from active virus protein.

DISCUSSION

It seems probable that the preparations previously described (1, 2), as well as those obtained by Bawden and Pirie (3) and those described in the present paper, represent latent mosaic virus protein free from the normal plant constituents present in infectious juice. However, from the evidence presented in this paper, there is good reason for distinguishing between preparations obtained by the ultracentrifugation of juice at low temperatures and those obtained by repeated precipitation with ammonium sulfate. The results show a great variation in relative or specific activity of the purified virus protein, depending on the type of treatment to which it had been subjected. Virus protein prepared by the ultracentrifugation of juice has, in general, possessed a uniform and high specific activity, whereas samples precipitated by ammonium sulfate or allowed to stand in ammonium sulfate solution have shown a variable and greatly decreased specific activity, as measured by their ability per unit of protein to produce primary lesions. The amount of virus activity lost has depended on the concentration of ammonium sulfate used and the length of time the virus was allowed to stand in contact with salt.

Bawden and Pirie recognized the low activity of the latent mosaic preparations which they obtained after ammonium sulfate treatment, as compared to that of the infectious juice, and suggested that the particles of virus had become irreversibly aggregated during the purification procedures to form rods. The evidence for aggregation, in addition to the loss of specific activity, was the failure of the purified virus to pass ultrafilters of 400 m μ average pore diameter (3, 24) and the fact that solutions of purified virus showed a large amount of stream double refraction, whereas

none was detected in the clarified juice itself. As all the latent mosaic preparations tested in the present work, whether prepared from juice by ultracentrifugation or after ammonium sulfate treatment, have been found to pass filters of about 400 m μ average pore diameter when in the presence of nutrient broth and phosphate at pH 8.5, it seems likely that those filtration results of Bawden and Pirie, in which water or dilute buffer alone was used as the solvent, can be explained by adsorption on the filter surface. Such a result would be expected in the absence of capillary-active substances (25). The evidence for an increase in stream double refraction is also questionable, because, as shown by Lauffer and Stanley, infectious juice from latent mosaic plants does show stream double refraction. In addition to these considerations, samples of low activity obtained after (NH₄)₂SO₄ treatment, as shown by Wyckoff,¹ have given a sedimentation diagram similar to that obtained from samples which have had no (NH₄)₂SO₄ treatment, except that the boundaries instead of being sharp and well defined have been relatively diffuse. It seems unlikely, therefore, that the loss of activity can be explained entirely on the basis of an irreversible aggregation. It may also be due to slight chemical changes in some of the virus particles. The analyses of samples prepared by different methods provide some evidence for this. Samples prepared by ultracentrifugation as compared with those obtained by Bawden and Pirie have shown differences in nitrogen, phosphorus, and carbohydrate content. The ratios of nitrogen to phosphorus and of nitrogen to carbohydrate are lower for samples prepared by ultracentrifugation of juice than those for samples obtained after repeated ammonium sulfate precipitation. This suggests that phosphorus and carbohydrate may have been lost after the chemical treatment. It might be expected that contact with strong electrolyte would cause some dissociation of protein and nucleic acid similar to that found for the simple nucleoprotein present in fish sperm, and that precipitation of the protein with salt might result in a loss of nucleic acid.

SUMMARY

The yields and relative or specific activity of latent mosaic virus prepared by chemical treatment and ultracentrifugation at low temperatures have been determined. The results show that

purified virus varied widely in relative activity, depending on the type of treatment used for purification. Repeated precipitation with salt or acid in general produced preparations of low and varying activity, as compared with virus obtained by ultracentrifugation, which possessed a uniform and high activity. The loss in activity was not accompanied by striking changes in the properties of the different preparations. They have the same qualitative solubility, the same serological properties, about the same ability to pass ultrafilters of about 450 m μ average pore diameter when dissolved in nutrient broth and 0.1 M phosphate at pH 8.5, and like the highly active virus form liquid crystalline solutions. They differ from the more active preparations in forming more opalescent solutions and in giving highly diffuse boundaries in the analytical ultracentrifuge. The loss in activity may be due partly to slight chemical changes which have taken place in such preparations.

The analyses of virus purified by ultracentrifugation are those of a nucleoprotein containing about 6 per cent nucleic acid. Qualitative tests for guanine and pentose and the isolation of a material with the qualitative solubility of yeast nucleic acid also show that a pentose nucleic acid is present. The ratio of carbohydrate to phosphorus is about twice that found by the same method of analysis for yeast nucleic acid; in addition to carbohydrate combined as nucleic acid other carbohydrate may therefore be present.

The latent mosaic virus is relatively unstable below pH 4 but stable over the range from pH 7.5 to 9, in contrast to tobacco mosaic virus which is stable below pH 4 but unstable between pH 7.5 and 9.

The relatively high homogeneity of the virus purified by the ultracentrifugation of clarified infectious juice, as shown by its uniform specific activity and the sharp boundaries obtained in the analytical ultracentrifuge, and the ability of the virus to pass ultrafilters of about 250 m μ average pore diameter indicate that such virus, in contrast to that prepared by treatment with ammonium sulfate, is essentially the same as that present in infectious juice.

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ACTION OF WHEAT AMYLASES ON SOLUBLE STARCH *

By OLOF E. STAMBERG AND C. H. BAILEY

(From the Division of Agricultural Biochemistry, University of Minnesota,
St. Paul)

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It was early discovered that at least two types of amylases exist, each capable of hydrolyzing starch, but in different manners. The mode of action by the two amylases has been subjected to many investigations. The saccharifying action is observed by reducing sugar determinations, the liquefying activity by the changes in viscosity of starch pastes. The dextrinizing activity may be followed by changes in the iodine color according to the method described by Wohlgemuth (1908), and also by the alcohol precipitation of the undextrinized starch, as shown by Caldwell and Hildebrand (1935).

β -Amylase, the saccharifying enzyme, produces an immediate appearance of maltose, while the iodine color reaction remains until most of the starch is saccharified. This enzyme is generally thought to hydrolyze maltose units from the ends of the starch or dextrin molecules. α -Amylase, the dextrinizing enzyme, apparently attacks the starch molecules at some internal linkages, thereby producing a rapid breakdown of the starch to the dextrin stage, resulting in an early disappearance of the iodine color reaction.

Several methods have been used in separating the amylases. Ohlsson (1926) used a procedure based on the thermolability of β -amylase and the acid lability of α -amylase. Klinkenberg (1932) employed an alcohol precipitation technique. Waldschmidt-

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Leitz and Purr (1931) used selective adsorption on colloidal aluminum hydroxide. Sherman, Caldwell, and Doebbeling (1934) fractionated the amylases by an ammonium sulfate precipitation. Holmbergh (1933) selectively adsorbed β -amylases on various starches, and in 1937 he described the preparation of α -amylase by rapid shaking of an alcoholic enzyme extract saturated with chloroform. Myrbäck and Ortenblad (1937) studied a variety of methods to purify and isolate the amylases from barley extracts. Caldwell and Doebbeling (1935) obtained two distinct amylases, an amylolytic and a saccharifying, from barley malt by the ammonium sulfate precipitation method.

EXPERIMENTAL

Two preparations of β -amylase and two of α -amylase from wheat were made by different methods. The β -amylase preparations were from normal wheat, and the α -amylase preparations were from germinated wheat. For the latter the wheat was steeped for 2 days at about 16° and then allowed to germinate at 20° for 3 days. The malted wheat was dried at 25°. The wheat samples were ground in a burr mill to a fine meal. 1 kilo of meal was extracted for 1 hour at 5° with 2.5 liters of aqueous 0.05 per cent NaH_2PO_4 solution.

One method used for the preparation of the amylases was that described by Klinkenberg (1932). α -Amylase was precipitated at 60 per cent concentration of alcohol by volume and β -amylase was precipitated at 80 per cent concentration from the respective extracts. The other method used was a combination of Ohlsson's (1926) and Klinkenberg's methods. According to Ohlsson the α -amylase is prepared by heating an extract to 70° for 15 minutes, thus inactivating most of the β -amylase present. β -Amylase is prepared by acidifying an extract with dilute HCl to pH 3.3 for 15 minutes at 0°, a treatment which inactivates most of the α -amylase. The method employed was to treat the respective extracts first according to Ohlsson's procedure and then precipitate the enzymes according to Klinkenberg's method.

All precipitates were washed with absolute alcohol and dried over CaCl_2 in a vacuum desiccator. The enzyme preparations made by Klinkenberg's method will be referred to as Amylase 1, and those with the combined Ohlsson and Klinkenberg methods

as Amylase 2. The yields of dry enzyme preparation based on the wheat meal used were 1.75 per cent α -Amylase 1, 0.7 per cent α -Amylase 2, 1.71 per cent β -Amylase 1, and 0.55 per cent β -Amylase 2.

A commercial diastase preparation, Merck's malt diastase, was used in addition to the prepared amylases. This will be referred to as malt diastase. Lintner soluble starch (Merck) was used throughout. A citrate buffer (Clark, 1920) at pH 5.1 was used, the final concentration in all hydrolysates being 0.02 N. The temperature used was 30°, except for the Wohlgemuth tests at 40°. The ferricyanide reduction method (Blish and Sandstedt, 1933) was used for all maltose determinations.

TABLE I
Results of Wohlgemuth and Caldwell-Hildebrand Tests on Enzyme Preparations

Enzyme	Wohlgemuth value*	Erythro-R value, per cent hydrolysis	Starch dextrinized, not precipitated in 55 per cent alcohol
			<i>per cent</i>
α -Amylase 2	214	15.3	64.4
“ 1	171	37.8	46.4
Malt diastase	100	44.4	40.4
β -Amylase 1	<1		7.4
“ 2	<1		3.7

* Expressed as mg. of starch hydrolyzed per mg. of enzyme.

Wohlgemuth Tests and Erythro-R Values—Wohlgemuth (1908) tests for the dextrinizing actions of the enzymes were made with 3 cc. of 2 per cent boiled soluble starch diluted with enzyme solution to 6 cc. and hydrolyzed for 1 hour. The value as expressed in Table I is in terms of mg. of starch hydrolyzed to the red-violet (erythro) end-point per mg. of enzyme. The reducing values reached at the end-point (the erythro-R values) in terms of per cent hydrolysis according to maltose determinations are also shown in Table I.

Caldwell-Hildebrand Tests—The dextrinizing activity of the enzymes according to the method of Caldwell and Hildebrand (1935) was determined on a 25 cc. portion of 2 per cent soluble starch paste which was hydrolyzed for 10 minutes at 30° with 10

mg. of enzyme. The percentage of starch dextrinized and not precipitable in 55 per cent alcohol is shown for each enzyme in Table I. Thus a higher value indicates higher dextrinizing activity of the enzyme.

Reducing Sugar Tests—The progress of the action of the enzymes was observed by reducing sugar determinations on 100 cc. of 1

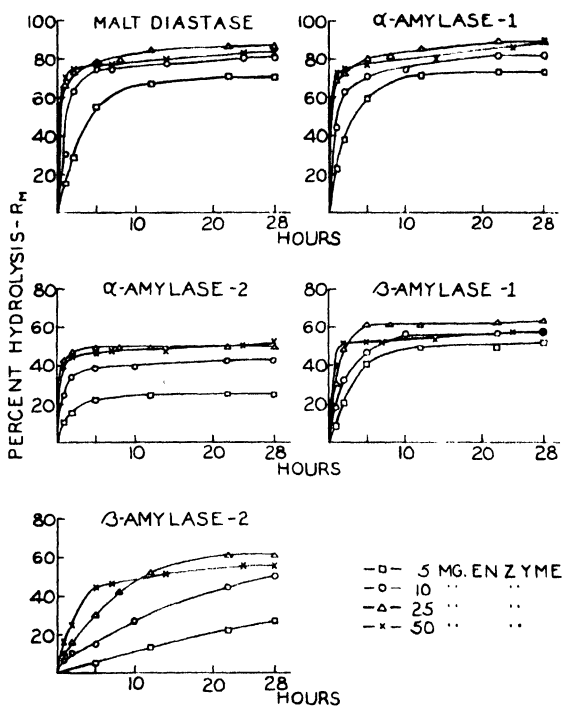


FIG. 1. Action of the enzymes on soluble starch at 30°, pH 5.1, with 5, 10, 25, and 50 mg. of each, as determined by the reducing sugar method (R_M) and expressed as per cent of available maltose.

per cent Lintner soluble starch. Cork stoppers were used to prevent evaporation and a few drops of toluene were added to each hydrolysate to prevent bacterial action. The rate of hydrolysis at various enzyme concentrations is shown graphically in Fig. 1.

α-Amylase 2 and β-Amylase 2, which apparently were quite pure as to their dextrinizing and saccharifying actions respectively,

were used for additional work. A 25 mg. portion of α -Amylase 2 was allowed to act on 100 cc. of 1 per cent boiled soluble starch solution for 12 hours. The hydrolysate was then divided into four aliquots and treated as follows: (1) this aliquot was boiled under a reflux for 1 minute to inactivate the α -amylase, and

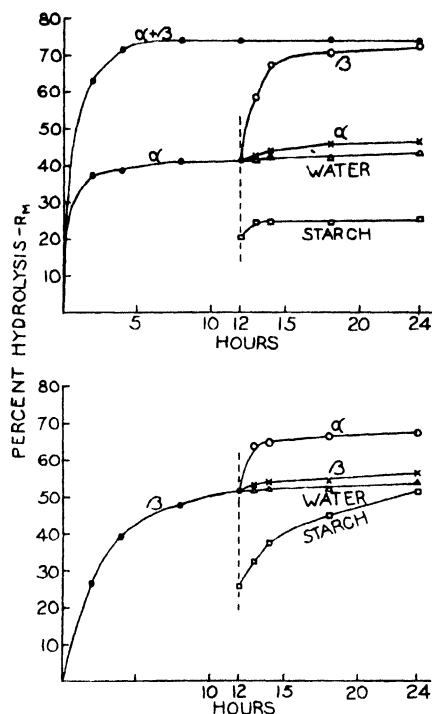


FIG. 2. Per cent hydrolysis (reducing sugar method) of α -Amylase 2 and β -Amylase 2 acting together, and each enzyme acting alone for 12 hours, after which aliquots were treated as indicated. The details are described in the text.

β -amylase was then added, the equivalent of 50 mg. per 100 cc. being used; (2) more α -Amylase 2, equivalent to 25 mg. per 100 cc., was added; (3) buffered water (pH 5.1) was added, thus diluting the system 1:1; (4) added 20 cc. of the original 1 per cent soluble starch solution. Another set of hydrolysates was made similarly but with 50 mg. of β -Amylase 2 for 12 hours, fol-

lowed by division of the hydrolysate into four aliquots. In one hydrolysate, 50 mg. of β -Amylase 2 and 25 mg. of α -Amylase 2 were used together. The results are shown graphically in Fig. 2, as obtained by maltose determinations and expressed as per cent hydrolysis.

Manometric Study of Action of Amylases—With 100 cc. of 1 per cent boiled soluble starch buffered at pH 5.1, 10 mg. of enzyme, and 2 gm. of starch-free yeast the CO_2 pressure was followed for 28 hours for each enzyme by means of flasks attached to manometers. The pressure was not allowed to rise above

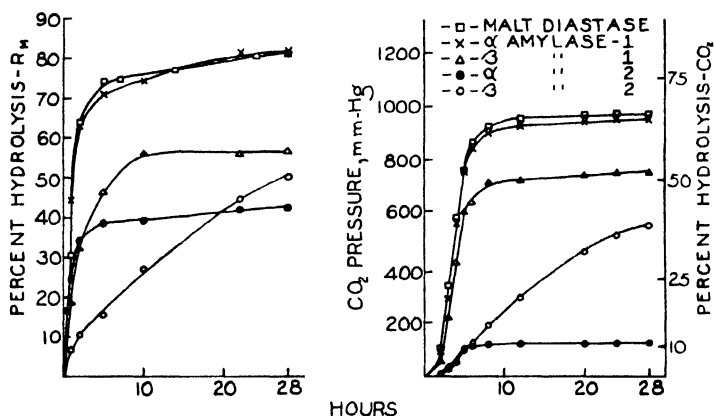


FIG. 3. Curves in the left-hand section were obtained by the reducing sugar method. The curves in the right-hand section give pressures obtained by the yeast-manometric method, with 10 mg. of enzyme in all cases. Per cent hydrolysis- CO_2 represents the estimated values at the respective pressures.

about 200 mm. of Hg before being released. The flasks were shaken until a constant pressure was obtained before each reading and release of pressure. Fig. 3 shows the pressure curves obtained and also the curves obtained by the reducing sugar method. A blank with the yeast and the starch suspension gave no pressure, an indication that the yeast preparation had no amylase activity.

The values in Figs. 3 and 4 expressed as per cent hydrolysis- CO_2 were obtained by the following method. Solutions of analyzed maltose were employed containing maltose equivalent to 10, 25, 50, and 75 per cent hydrolysis, respectively, of the 1 per cent starch

solution. To these were added 2 gm. of fresh yeast, and 0.5 gm. of dried yeast to supply the activator described by Blish and Sandstedt (1937). At the end of the hydrolysis only traces of reducing substances remained, indicating the maltose was completely fermented. The concentrations of the maltose solutions were determined by reducing sugar tests. The method was only an attempt to estimate the relation between the pressure values and the per cent hydrolysis by the reducing sugar method with the particular system used. The total pressures obtained for maltose

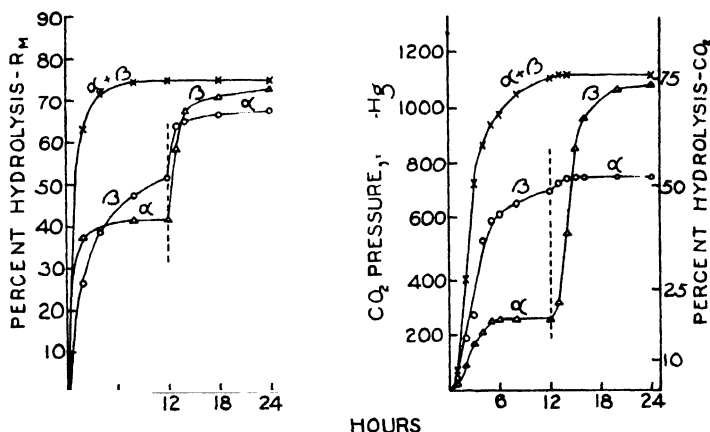


FIG. 4. The action of 25 mg. of α -amylase and 50 mg. of β -amylase together, first 25 mg. of α -amylase then 50 mg. of β -amylase, and first 50 mg. of β -amylase then 25 mg. of α -amylase as followed by the reducing sugar method (R_M) and the yeast-manometric method (CO_2). α -Amylase 2 and β -Amylase 2 were used.

equivalents of 10, 25, 50, and 75 per cent hydrolysis of the starch solution were 107, 362, 728, and 1106 mm. of Hg, respectively.

Fig. 4 shows graphically the results of a series of tests on the purer amylases (α -Amylase 2 and β -Amylase 2) under similar conditions as for Fig. 3. Each enzyme acted alone, since the solutions were boiled under a reflux for 1 minute before the addition of the other enzyme.

DISCUSSION

The various tests applied to the enzyme preparations indicate that both β -amylase preparations were quite free from α -amylase.

This is in agreement with Nord and Ohlsson (1932) and Andrews and Bailey (1934) that the chief enzyme in normal grain is β -amylase. Both of these enzymes hydrolyzed soluble starch to about 60 per cent (reducing sugar method) leaving the erythro-granulose portion. This has been observed by most workers who employed pure β -amylase.

α -Amylase 2 was the most active dextrinizing enzyme, as shown by the high Wohlgemuth value, an erythro-R value of 15.3 per cent, and the greater dextrinizing action, as determined by the alcohol precipitation method. This enzyme hydrolyzed the soluble starch to about 40 per cent, as determined by the reducing sugar method. α -Amylase 1 had less dextrinizing action and appeared to have appreciable quantities of β -amylase present, as shown by the higher erythro-R value of 37.8 per cent and the ability to hydrolyze soluble starch paste to about 89 per cent. Malt diastase had slightly less dextrinizing action than α -Amylase 1, but the latter two enzymes acted much alike, as measured by the reducing sugar method.

The graphs in Fig. 2 show that when α -amylase was allowed to act first, followed by β -amylase, about the same amount of hydrolysis was obtained as when both enzymes acted together. This confirms the conclusion of Ohlsson (1926) that α -amylase produces mainly dextrans which then serve as substrate for β -amylase. When β -amylase was allowed to act first to 52 per cent hydrolysis, the α -amylase would then proceed to about 67 per cent, an increase of only 15 per cent. Thus Klinkenberg's theory that starch has 36 per cent α starch attacked only by α -amylase and 64 per cent β starch attacked only by β -amylase appears erroneous. Hanes (1935) concluded that the amylases do not show the specificity of action proposed by Klinkenberg. Freeman and Hopkins (1936) likewise concluded that α - and β -amylases attack most readily the same fractions of the starch. The addition of more enzyme or the dilution of the system did not increase the rate of action appreciably after the respective enzymes had acted for 12 hours. Addition of more starch solution showed that the enzymes were still active in the hydrolysates after 12 hours. β -Amylase appeared to remain much more stable than the α -amylase after 12 hours under the conditions.

The yeast-manometric method used in this study in combination with the reducing sugar method proved useful in demonstrating

that α -Amylase 2 was quite free from the β enzyme, and that α -amylase produces mainly non-fermentable reducing dextrins which are easily saccharified by β -amylase. α -Amylase 2 did not produce fermentable reducing sugars equivalent to the per cent hydrolysis by the reducing sugar method, and as estimated from the CO_2 pressure values only about 12 per cent was due to fermentable sugars, while the remaining 28 to 30 per cent was due to non-fermentable reducing substances. Blom, Braae, and Bak (1938) using a commercial bacterial amylase high in α -amylase, showed that not all of the products formed during the early stages of starch hydrolysis were fermentable sugars, as indicated by the difference in reducing value of the hydrolysates before and after the action of yeast. Fig. 4 gives additional evidence that pure α -amylase does not produce fermentable reducing sugars equivalent to the per cent hydrolysis by the reducing sugar method. Addition of β -amylase following the action of α -amylase produced a rapid appearance of fermentable sugars. The addition of α -amylase after β -amylase had acted showed that although reducing groups appeared there was very little fermentable material.

SUMMARY

Two α -amylase preparations were made from germinated wheat, the first by direct alcohol precipitation, and the second by a heat treatment of the extract followed by alcohol precipitation. These are referred to here as α -Amylase 1 and α -Amylase 2, respectively. Two β -amylase preparations were made from normal wheat, the first by direct alcohol precipitation, the second by an acid treatment of the extract followed by alcohol precipitation, referred to as β -Amylase 1 and β -Amylase 2, respectively.

Both β -Amylase 1 and β -Amylase 2 were quite pure (free from α -amylase), indicating that normal wheat contains primarily β -amylase. α -Amylase 2 was a very active and quite pure dextrinizing amylase, while α -Amylase 1 appeared to be a mixture and acted much like commercial malt diastase.

Hydrolysis of soluble starch by the β -amylase preparations was about 60 per cent, by α -Amylase 2 about 40 per cent, and by α -Amylase 1 and malt diastase about 85 per cent, as determined by the reducing power in terms of available maltose. α - and β -amylase apparently attacked the same portion of the starch.

With a yeast-manometric method and soluble starch as sub-

strate, it was apparent that the pure α -Amylase 2 produced mainly non-fermentable reducing dextrins together with some fermentable reducing sugars. The other enzyme preparations produced fermentable reducing sugars equivalent to the reducing value.

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REVERSIBLE ACTION OF OXIDIZED PHENOLS IN THE DEAMINATION OF CERTAIN AMINO ACIDS

By STEPHEN S. HUBARD

(From the Department of Chemistry, Cornell University, Ithaca)

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Robinson and McCance (1), in reporting work confirming the conclusions of Happold and Raper (2) that a phenolic substance is necessary for the oxidative deamination of amino acids in the presence of tyrosinase, suggest that their results indicate a coupled reaction of some sort for the breakdown of the acids. Subsequent work has shown that this is so. Phenolic substances which can function in such deaminations are converted into *o*-quinones, and these oxidatively deaminate amino acids in the absence of the enzyme (2, 3). The rôle of the latter is to oxidize the phenolic substance to a form that can react with the amino acid, and not to bring about the breakdown directly. No systematic data exist to indicate whether the quinone intermediate can function reversibly if the amount of amino acid is too great to be entirely consumed in a primary reaction with it. Presumably, the *o*-quinone is reduced by the amino acid to an *o*-dihydroxylic compound, which might then be enzymically reoxidized to a quinone, and so react with more of the amino acid. On the other hand, it might be removed from the sphere of reaction by combining with the end-products; both processes might take place to some extent.

Accordingly, solutions containing fixed amounts of tyrosinase and glycine with varying amounts of *p*-cresol were aerated and the extent of deamination determined.

EXPERIMENTAL

If the quinone group of the oxidized phenol is reduced by 2 hydrogen atoms from the amino acid, then the phenol and the acid are equivalent mole per mole. On this basis, 72 mg. of *p*-cresol are equivalent to 50 mg. of glycine and are the amount

of *p*-cresol used as a reference in planning our study. Erlenmeyer flasks of 125 cc. capacity containing 50 mg. of glycine dissolved in 10 cc. of a phosphate buffer solution adjusted roughly to pH 7.8, 10 cc. of a tyrosinase extract prepared essentially according to the method of Graubard and Nelson (4), with a trace of toluene as a preservative, and the various quantities of *p*-cresol listed in Table I were plugged with cotton and kept in a 20° incubator for 72 hours, with occasional shaking to saturate the solutions with oxygen.

After 3 days, the ammonia was distilled from each solution following the addition of excess calcium hydroxide, and a standard

TABLE I
Deamination in Presence of Varying Amounts of p-Cresol

Deter- mination No.	<i>p</i> -Cresol used	Amino N found	Glycine deaminized	Deamina- tion expected if <i>p</i> -cresol acted irre- versibly	N recovered as ammonia	Actual re- covery of ammonia in terms of ex- pected, based on Van Slyke data
	mg.	mg.	per cent	per cent	mg.	per cent
Blank	0	9.34	0	0	0	
1	1	7.04	24.6	1.4	2.38	103.5
2	2	6.56	29.8	2.8	2.52	90.7
3	4	5.67	39.2	5.6	3.29	87.3
4	12	3.57	61.7	16.7	4.20	72.8
5	36	1.58	83.1	50.0	4.38	56.5
6	72	1.36	85.4*	100.0*	4.51	56.5
7	144	1.31	85.9*	100.0*	4.33	53.9

* See "Discussion."

Van Slyke determination of the amino nitrogen was made. The data and results are given in Table I.

DISCUSSION

The results show clearly that the glycine in the flasks containing less than the equivalent of *p*-cresol undergoes oxidative deamination to an extent considerably beyond that expected if the *o*-quinone group were not regenerated by enzymic oxidation. The figures might have been even higher if agitated manometers had been used, for the reaction did not go to completion in the solutions containing an excess of *p*-cresol, probably because the oc-

casional shaking did not furnish sufficient oxygen while the enzyme was still active.¹

The reversibility is not complete; that is, the smaller amounts of *p*-cresol do not effect the oxidation of as much glycine as do the larger ones. Apparently, while the main reaction is in progress, a part of the oxidized *p*-cresol is removed by reaction with one or more of the end-products of the deamination. This seems the more probable when it is noted that less than the expected amount of ammonia is recovered, and that the loss is greater the greater the percentage deamination. Our results agree with those of Pugh and Raper (3) in showing this incomplete recovery.

If the oxidized *p*-cresol functions reversibly, then the amount of oxygen needed to regenerate it to the extent consistent with the amount of deamination in a particular case should agree with the amount of oxygen consumed by that solution in excess of what is taken up by a similar one containing *p*-cresol without glycine. This is supported by data of Robinson and McCance (1). For 2.5 mg. of *p*-cresol to deaminize almost completely 4.5 mg. of glycine, 620 c.mm. more of oxygen should be used by the system when the glycine is present than when it is absent. Robinson and McCance found 1255 c.mm. with glycine, 615 c.mm. without it, giving a difference of 640 c.mm., which agrees well with our calculated value of 620 c.mm., based upon 1 atom of oxygen per mole of oxidized *p*-cresol to convert the *o*-dihydroxyl group to an *o*-quinone.

Since catechol acts in a deamination by means of the *o*-quinone group in its oxidized form, it should also be expected to function reversibly. Results of Robinson and McCance can be explained on this basis. A calculation corresponding to that in the preceding paragraph gave 397 c.mm., against an observed value of 380 c.mm. (difference between 790 and 410). Leucine and *p*-cresol gave 488 c.mm., calculated, and 540 c.mm., observed. The data for alanine with *p*-cresol and glycine with resorcinol were insufficient for such comparisons.

SUMMARY

1. *p*-Cresol functions reversibly in the oxidative deamination of glycine through the enzymic regeneration of the *o*-quinone group

¹ Robinson and McCance found 94.5 per cent of their glycine deaminized (calculated by us from their data).

in its oxidized form, after the latter has reacted with some of the glycine.

2. A very small amount of *p*-cresol does not take care of an unlimited amount of glycine, owing probably to the removal of part of the oxidized *p*-cresol to form a complex with some of the end-products, simultaneously with the main reaction.

3. Catechol with glycine and *p*-cresol with leucine probably function reversibly also, since the data of Robinson and McCance are consistent with this theory.

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RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM

IV. THE PHOSPHOLIPID METABOLISM OF THE BRAIN*

By G. W. CHANGUS, I. L. CHAIKOFF, AND S. RUBEN

(From the Division of Physiology of the Medical School and the Department of Chemistry, University of California, Berkeley)

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Phospholipid is abundant in the brain, yet little is known of its metabolic activity there. McConnell and Sinclair (1) employed elaidic acid for comparing the phospholipid turnover of various tissues. They found that brain incorporates elaidic acid into cephalins and lecithins more slowly than muscle or liver. In previous reports from this laboratory (2-4), in which the new indicator, radioactive phosphorus, was used for labeling phospholipids, it was shown that radioactive phospholipids are more slowly deposited or synthesized by brain than by kidney, liver, or gastrointestinal tract. Similar observations have been made by Hahn and Hevesy (5).

In the present study, the rate of phospholipid turnover was studied in rat brains for several weeks after the administration of radioactive phosphate. It is shown that, despite the slow turnover, brain continues to increase its net content of radioactive phospholipid for several days after the ingestion of radioactive phosphate and that, once the maximum amount has been deposited, very little loss occurs even as late as 3 weeks after. The sensitivity of the radioactive indicator method in following phospholipid metabolism in tissues is particularly well brought out by the present investigation, for amounts as low as 0.01 per cent of the labeled phosphorus fed were measured after its incorporation into phospholipid.

* Aided by grants from Child Neurology Research (Friedsam Foundation) and from the Research Board of the University of California.

EXPERIMENTAL

The data recorded in this study were obtained from 159 rats. Experiments were conducted on several groups of animals, these

TABLE I
Phospholipid Metabolism of Brain

Group No.	Symbol	Chart No.	No. of rats	Weight*		Fast period before P ³² administration	Phosphorus fed†			Treatment after P administration
				Range	Average		Volume	P as NaHPO ₄	Radioactive units† × 10 ⁶	
				gm.	gm.	hrs.	cc.	mg.		
1§	○	1	34	232-360	289	26	2	42.2	1.5	Fasted throughout
2	⊙	1	20	120-162	144	26	2	5.0	4.0	“ “
3	●	1	20	120-162	144	26	2	5.0	4.0	“ 26 hrs., then given access to food
4	⊕	1	4	116-160	137	24	1	0.5	4.0	Fasted 24 hrs., then sacrificed
5	◦	1	14	110-150	138	26	2	10.0	7.0	Fasted 26 hrs., then access to food
6	□	1	4	100-118	106	26	1	4.2	8.0	“ “
7	×	1	4	206-240	228	26	1	4.2	8.0	“ “
8	●	1	8	58- 75	67	17	1	4.2	8.0	Fasted 24 hrs., then access to food
9	○	2	30	28- 32	30	15	1	0.5	4.0	Fasted 20 hrs., then access to food
10	◦	2	13	32- 40	39	17	1	4.2	8.0	Fasted 24 hrs., then access to food
11	●	2	8	38	38	18	1	2.8	8.0	Fasted 15 hrs., then access to food

* At the time of P³² administration.

† All feedings of labeled P given by stomach tube.

‡ 1 radioactive unit ~ 2×10^{-12} curie.

§ Seventeen rats of this group received 2 cc. of olive oil just previous to the P³² administration.

varying in weight as well as nutritional state. The various series of animals studied are shown in Table I and Figs. 1 to 3.

Each rat received by stomach tube 1 or 2 cc. of a solution of Na₂HPO₄ containing between 0.5 and 42 mg. of phosphorus. All animals had been fasted for 15 to 26 hours beforehand. The

subsequent treatment varied; some were fasted throughout, while others were allowed access to food after the phosphate was fed. Both adult and young rats were investigated. The results obtained are shown in Figs. 1 and 2, respectively. All intervals shown in Figs. 1 to 3 refer to the time after the animals received the labeled phosphorus. Each point represents the mean value for radioactive phospholipid obtained from separate determinations on the brains of four rats. Labeled phospholipids were followed for as long as 800 hours after the administration of the radioactive phosphorus.

Treatment of Brain Tissue—The animals were sacrificed by decapitation and the brain removed immediately thereafter. Care was taken to insure uniformity in the amount of tissue excised. The brain, including the olfactory lobes, was raised from the floor of the cranium and the nerves cut close to the base. The hypophysis was not included. The brain was then severed from the cord by section at the level of the first cervical nerve and immediately placed in 50 cc. of 95 per cent ethyl alcohol and refluxed for 2 hours at approximately 55°. The solvent was then decanted and the brain residue finely minced and again extracted once or twice by refluxing for 2 hours with fresh portions of 50 cc. of ethyl alcohol. The residue thus obtained was placed in a Soxhlet apparatus and subjected to continuous extraction for 12 hours, the first 6 with ethyl ether, the second 6 with petroleum ether. The alcohol, ethyl ether, and petroleum ether extracts were combined and concentrated *in vacuo* at 55° to a volume of approximately 5 cc. in the presence of a stream of CO₂. The lipids were extracted from this concentrate with several washings of petroleum ether in a separatory funnel and the phospholipids precipitated in a manner previously described (6). The method by which the phospholipids were mounted and their radioactivity determined has been noted elsewhere (2).

In two groups of rats the brain residues obtained by this treatment were subjected to further extraction with five portions of 20 cc. of hot chloroform. In the case of one group a carrier in the form of soy bean lecithin was added to the chloroform extracts and the radioactivity measured in the phospholipids isolated by precipitation with acetone and magnesium chloride (6). In the other group the radioactivity was determined in the entire

chloroform extract. The radioactivity of the extracts prepared from the brain residues of both groups was negligible in comparison with that found before extraction with chloroform.

Results

The results obtained with eleven groups of rats are shown in Figs. 1 and 2. In the former are recorded the values for radioactive phospholipid found in the older rats; Fig. 2 contains the values for the younger group of rats.

Radioactive phospholipid slowly accumulates in the brains of both young and adult rats, the amount deposited by both groups

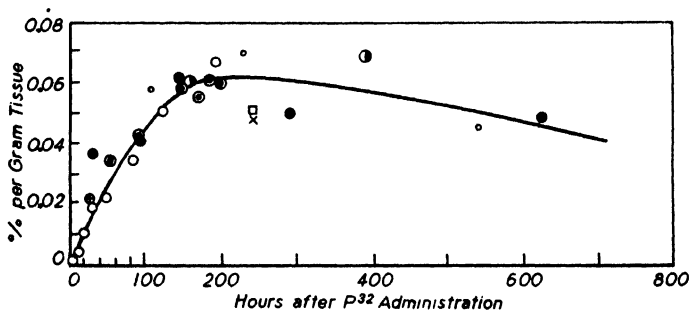


FIG. 1. The labeled phospholipid content per gm. of adult brain. The ordinates represent the per cent of administered phosphorus found as phospholipid per gm. of brain tissue. For the meaning of the symbols see Table I. Each point is the mean of four separate determinations on the brains of four rats.

reaching a maximum at about 200 to 250 hours after the administration of the labeled phosphorus (Figs. 1 and 2). The amounts deposited in the brains of the two age groups, however, differ considerably (Fig. 3). At the 200 hour interval, 0.06 per cent of the labeled phosphorus was found in the phospholipids of each gm. of adult brain, while in the younger animals 0.14 per cent was present. Thus, at the height of the curves, the younger brain shows an activity more than twice as great as the adult. This increased phospholipid activity is apparently a true characteristic of the brain of the immature animal, for although changes in water content have been shown to occur in the growing brain, these do not exceed 5 per cent in the age periods studied here (7).

This is in keeping with previous findings in which it was shown that the rate at which phospholipids are deposited is greater in the young than in the adult rat (1, 8).

The slow rise of labeled phospholipid in brain tissue stands in

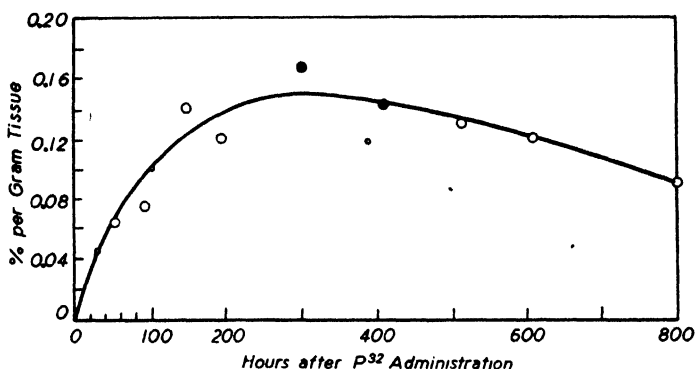


FIG. 2. The labeled phospholipid content per gm. of young brain. The ordinates represent the per cent of administered phosphorus found as phospholipid per gm. of brain tissue. For the meaning of the symbols see Table I. Each point is the mean of four separate determinations on the brains of four rats. These animals weighed 28 to 38 gm. at the time of receiving the radioactive phosphorus.

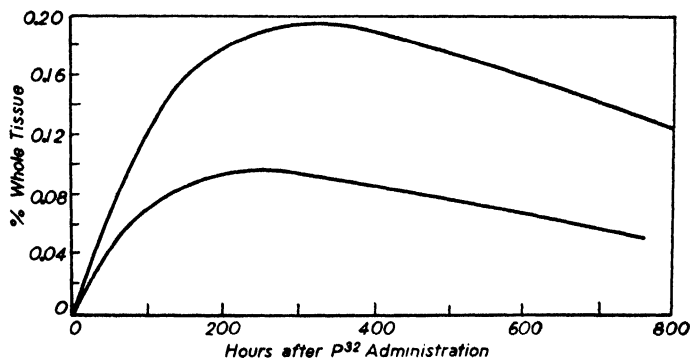


FIG. 3. The comparative labeled phospholipid metabolism in the whole brains of young and adult rats. The ordinates represent the per cent of administered phosphorus found as phospholipid in the entire brain. The lower curve was obtained from the older group of rats and the upper from the younger rats.

marked contrast to that of three other tissues—liver, kidney, and small intestine—in which it reaches a high peak within 10 to 20 hours after the administration of radioactive phosphorus and then rapidly falls (2). After attaining a maximum concentration in the brain, the radioactive phospholipid decreases very slowly. Thus, 625 hours after the administration of the radioactive phosphorus the brains of adult rats still contained 70 per cent of the amount deposited at the 200 hour interval. The course of events in the brains of the younger animals is equally striking. At such widely differing intervals as 200 and 800 hours after the phosphorus ingestion, their brains contained respectively 0.14 and 0.10 per cent of the fed phosphorus as labeled phospholipid per gm. of tissue. At the 800 hour interval their brains still contained 70 per cent of the amount present at the 200 hour interval.

The rate of phospholipid turnover in the brain is of particular interest in connection with its selection of fatty acids in forming phospholipids. The slower incorporation of elaidic acid by brain than by other tissues, such as liver, has led to the conclusion that brain is more selective in the fatty acids used in building up its phospholipids (1). The present study shows that the phospholipid turnover of the liver is many times that of the brain, and so it is to be expected that, in the presence of a foreign fat, the fatty acid pattern of liver phospholipid would alter much more rapidly than that of brain phospholipid. Hence it is not at all unlikely that the brain's more rigorous selection of fatty acids used in the formation of phospholipids may be merely the result of its particular rate of phospholipid turnover. It must be recognized, however, that the present study does not necessarily exclude the possibility that tissues exhibit differences in their ability to select fatty acids for the synthesis of phospholipids.

The results of the present study demonstrate quite clearly that the phospholipid *content* of a tissue bears no relation to its phospholipid *turnover*. Despite the fact that brain is particularly rich in phospholipids, containing, as it does, perhaps twice as much as liver or kidney, its phospholipid turnover, as judged by the formation of radioactive phospholipid, is but a fraction of that occurring in those tissues.

Variations in nutritional state failed to produce measurable effects on the phospholipid activity of the brains of adult animals. Although the rats of Group 2 were fasted and those of Group 3 were fed throughout the period of study, the radioactive phospholipid content of both groups was practically identical after the ingestion of the same dose of labeled phosphorus. In this respect, brain again differs from liver and the small intestine, in both of which phospholipid activity was influenced by the ingestion of oil. It is also interesting to note that rats of Group 1 were fed 42 mg. of labeled phosphorus, whereas those of Groups 2 and 3 received 5 mg.; yet a constant percentage of the labeled phosphorus fed was deposited as brain phospholipid (Fig. 1). The values for radioactive phospholipid of all three groups lay on the same curve. This is not surprising, since the 5 and 42 mg. of labeled phosphorus become intimately mixed with the relatively greater amounts of unlabeled phosphorus already present in the body.

SUMMARY

1. The phospholipid metabolism of the rat brain was investigated with radioactive phosphorus as indicator.

2. Following the ingestion of labeled phosphate, brain shows a much slower deposition of radioactive phospholipid than liver, kidney, or small intestine. A progressive increase in the content of radioactive phospholipid was observed as long as 200 hours after the administration of the phosphorus. Once the maximum amount of labeled phospholipid has been deposited, its loss from the brain occurs at a very slow rate. Radioactive phospholipid was found as late as 4 weeks after the labeled phosphorus was fed.

3. The rate of formation of brain phospholipids is identical in the fed and fasted rat.

4. The brain of the young rat incorporates radioactive phospholipid in greater amounts than the brain of the adult.

We are indebted to Professor E. O. Lawrence and members of the Radiation Laboratory for the radioactive phosphorus used in this study. It was prepared by bombardment of phosphorus with deuterons accelerated in the cyclotron. Assistance was also furnished by the Works Progress Administration.

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THE METHYLATION OF URSOLIC ACID

BY HAROLD M. SELL AND ROLAND E. KREMERS

(From the Research Laboratory, General Foods Corporation, Battle Creek)

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The literature dealing with the methylation of ursolic acid (1-7) indicates that disagreement exists among various investigators on the identity of methyl ursolate ($C_{30}H_{48}(OH)COOCH_3$) obtained. This might be ascribable to variations in the purity of the ursolic acid used by the different investigators or to the influence of the reagents and conditions employed in the methylation.

In 1933, Kuwada and coworkers (6, 7) reported two methylation products of ursolic acid which they considered to be isomeric forms of methyl ursolate. Product I (m.p. 230° , 3.4 per cent OCH_3 , theory 6.6 per cent OCH_3 , slightly soluble in acetone) and Product II (m.p. $170-172^\circ$, 5.93 per cent OCH_3 , soluble in acetone) were obtained upon treatment of ursolic acid with diazomethane, methyl iodide-silver oxide, or alkali and dimethyl sulfate.

By the action of diazomethane on crude ursolic acid, Jacobs and Fleck (5) obtained two products, one which melted at $221-222^\circ$ (3.3 per cent OCH_3 , slightly soluble in acetone) and another product which melted at $110-120^\circ$ (6.3 per cent OCH_3 and soluble in acetone). The acetone-soluble product was identical with the previously known methyl ursolate monohydrate. They regarded the higher melting product as being "possibly a molecular association compound of ursolic methyl ester and some other substance of the same molecular order" and not as an isomeric ester.

In view of the analytical results recorded by both groups of investigators, the interpretation of Jacobs and Fleck (5) appears to be more rational than that advanced by Kuwada and coworkers (6, 7). In order to settle the issue raised by these conflicting reports, methyl ursolate was prepared by the three different methods of methylation described above and the reaction products

were carefully examined. All reactions were carried out with purified ursolic acid, m.p. 283–284°, which yielded derivatives of accepted constants. After methylation, the unchanged material was removed from the methyl ursolate.

In our experiments the same methyl ester was obtained by all three procedures. The solubility of the product in acetone and the excellent yields of 94 to 97 per cent of the methyl ester, m.p. 170–171°, show that none of the so called high melting product of the Japanese authors was formed. Until it can be shown that an isomeric methyl ester can be obtained from a definitely characterized isomeric acid, we are inclined to believe that only one true methyl ester of ursolic acid exists. This is the ester which melts at 170–171° when anhydrous and has an indefinite melting point when it contains water of crystallization.

EXPERIMENTAL

Methylation of Ursolic Acid with Dimethyl Sulfate—To 3 gm. of ursolic acid dissolved in 50 cc. of absolute methanol were added 6.58 cc. of normal alcoholic potassium hydroxide and 1.0 gm. of redistilled dimethyl sulfate. The mixture was refluxed for 3 hours, poured into water, and then extracted with ether. The ether solution was washed three times with 100 cc. portions of cold 3 per cent sodium hydroxide solution and once with 100 cc. of water, and evaporated; the residue, dried on a steam bath, weighed 2.9 gm. (94 per cent of the theoretical) and had a melting point of 169–170°. The ester was recrystallized from 70 per cent ethanol and air-dried overnight. The melting point was indefinite above 110° and the loss of weight at 111° and 5 mm. was 4.23 per cent. The anhydrous product melted at 170°.

Methylation of Ursolic Acid with Diazomethane—To 3 gm. of ursolic acid were added 5 gm. of diazomethane in 100 cc. of ether. The reaction was allowed to proceed until solution was complete. The ether and diazomethane were removed by evaporation. The product was redissolved in ether and treated in the same manner as the product from dimethyl sulfate. The yield was 2.9 gm. or 94 per cent of the theoretical; m.p. 170–171°. The melting point after recrystallization from 70 ~~per~~ cent ethanol was indefinite above 110° and the loss of weight at 111° and 5 mm. was 4.5 per cent. The final product melted at 171°.

Methylation of Ursolic Acid with Methyl Iodide—The silver salt prepared from 3 gm. of ursolic acid was placed in a flask with 20 cc. of absolute methanol, 33 cc. of redistilled methyl iodide, and 35 cc. of ether, and refluxed for 3 hours. The precipitated silver iodide was removed by filtration and the solvents removed by evaporation. The residual methyl ester was taken up in ether and purified as previously described. The yield was 3 gm. or 97 per cent of the theoretical; m.p. 169.5°. After recrystallization from 70 per cent ethanol, the melting point was indefinite above 110° and the loss of weight on drying was 4.35 per cent. The final melting point was 170°.

SUMMARY

Ursolic acid was esterified with diazomethane, methyl iodide (silver ursolate), and dimethyl sulfate. The same ester, m.p. 170–171°, was isolated in each instance. This is not in harmony with the reports of Kuwada and coworkers who claim that there are two isomeric esters of ursolic acid.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LIV. THE ISOLATION AND PROPERTIES OF MYCOLIC ACID*

By F. H. STODOLA,† ALEX LESUK,‡ AND R. J. ANDERSON

(From the Department of Chemistry, Yale University, New Haven)

(Received for publication, August 13, 1938)

In the analysis of the purified wax (1) isolated from the human tubercle bacillus it was found that the principal ether-soluble constituent was a hydroxy acid of very high molecular weight which was designated by the term "unsaponifiable wax" (2). This substance has been the subject of further studies, the results of which are reported in this paper.

The so called waxes of the human tubercle bacillus are exceedingly complex mixtures and they show great differences in composition. Some of the wax fractions that we have examined contain large quantities of carbohydrates, for instance the purified wax (1) and the unfiltrable lipid (3); again, other fractions, such as the soft wax (4), are complex glycerides. The carbohydrate components when present are easily split off by treatment with alcoholic potassium hydroxide, the wax being thus separated into two fractions, (a) water-soluble carbohydrate and (b) ether-soluble compounds. The ether-soluble components consist of a mixture of fatty acids and incompletely saponified wax-like material. The waxy substance can be separated from the fatty acids by precipitation from ether solution with alcohol. The alcohol-insoluble material is extremely stable but on prolonged saponi-

* The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of a National Tuberculosis Association Fellowship at Yale University, 1936-37.

‡ Holder of a National Tuberculosis Association Graduate Fellowship at Yale University, 1937-38.

fication it yields fatty acids and the dihydroxy monomethoxy alcohol, phthiocerol (5). The chief component, however, is the hydroxy acid with properties very similar to those of the "unsaponifiable wax."

In view of the fact that the hydroxy acid of high molecular weight is the principal ether-soluble constituent of all the wax fractions of the human tubercle bacillus and further that analogous hydroxy acids form the chief ether-soluble components of the waxes of other acid-fast bacteria, it seems desirable to designate these peculiar acids by some distinctive name. We propose, therefore, to name the hydroxy acid from the human tubercle bacillus wax mycolic acid. Analogous acids from other acid-fast bacteria may then be designated by adding some appropriate prefix to mycolic acid.

The properties of mycolic acid differ very slightly from those described for the "unsaponifiable wax." Mycolic acid is acid-fast. It is a saturated acid with a low dextrorotation and it contains hydroxyl and methoxyl groups. The simplest formula calculated from its composition is $C_{88}H_{172}O_4$ or $C_{88}H_{176}O_4$ on the assumption that it is a monobasic acid. It may, however, be a dibasic acid having a molecular weight of about 2600.

EXPERIMENTAL

In the examination of a wax fraction of the human tubercle bacillus described in Paper XLVI of this series (5) Stodola and Anderson found that the principal cleavage products were (a) a material whose properties corresponded to the so called "unsaponifiable wax," (b) certain fatty acids of high molecular weight, and (c) the dihydroxy monomethoxy alcohol, phthiocerol. The wax was very stable and required about 80 hours of refluxing in benzene solution with methyl alcoholic potassium hydroxide before saponification was complete. After this prolonged alkali treatment further refluxing in the same manner for 65 hours gave no additional cleavage products.

The so called "unsaponifiable wax" described by one of us (2) several years ago had not been subjected to such prolonged saponification and for this reason might be incompletely saponified. A portion of the original "unsaponifiable wax" was therefore re-

saponified by refluxing in benzene solution with methyl alcoholic potassium hydroxide for several days.

The reaction mixture was worked up as follows: The alkaline solution was transferred to a separatory funnel, mixed with ether, diluted with water, and acidified with hydrochloric acid. After thorough shaking the aqueous layer was drawn off and discarded. The ether-benzene solution, after being washed free of acid with water, was dried over sodium sulfate and evaporated to dryness. The residue was dissolved in 200 cc. of ether and 400 cc. of alcohol were added, after which the solution was cooled, whereupon a white precipitate separated which was filtered off and washed with alcohol. The filtrate was concentrated until most of the ether had been removed, and allowed to cool. A white precipitate which separated was collected and added to the first lot.

The filtrate was further concentrated and mixed with an alcoholic solution of lead acetate. The lead salt was filtered off and decomposed in ethereal suspension with dilute acid. The fatty acid was obtained on evaporation of the ether.

The filtrate from the lead salt was concentrated nearly to dryness, diluted with water, acidified with dilute nitric acid, and extracted with ether. The ethereal extract was washed with water, after which it was washed with dilute potassium hydroxide in order to remove such fatty acids as had not been precipitated by lead acetate. The ethereal extract was again washed with water, dried over sodium sulfate, and evaporated to dryness. The residue thus obtained consisted of neutral material, *i.e.* crude phthiocerol. From the alkali washings mentioned above a small amount of fatty acids was recovered and combined with the acids obtained from the alcohol-insoluble lead salts.

A portion of the "unsaponifiable wax" weighing 32.2 gm. yielded the following products.

	gm.
Ether-alcohol-insoluble material.....	30.3
Total fatty acids.....	1.1
Crude phthiocerol.....	0.6

The results indicate that additional cleavage products had been liberated by the alkali treatment. The fatty acids were not examined, but the neutral material, phthiocerol, has been described in a former paper (5).

Examination of Mycolic Acid

The ether-alcohol-insoluble material which resembled the so called "unsaponifiable wax" in properties will be referred to hereafter by the name mycolic acid. For purification the substance was repeatedly precipitated from ethereal solution by addition of 2 volumes of alcohol. Mycolic acid always separated as fine, colorless globular particles either from acetone, ether, or ether-alcohol solutions, and it could not be obtained in crystalline form. The melting point of different preparations was 54–56° (corrected). The equivalent weight was determined by titrating a solution of mycolic acid in moist ether with standard alcoholic potassium hydroxide, phenolphthalein being used as indicator. The values obtained ranged from 1270 to 1316. The average value of seven different preparations was 1284.

Molecular Weight (Rast)—Found 1117. The methyl ester of mycolic acid gave values of 1111 and 1101.

Rotation—0.7133 gm. of substance dissolved in chloroform and diluted to 10 cc. gave in a 1 dm. tube a reading of +0.13°; hence $[\alpha]_D^{25} = +1.8^\circ$.

It was reported by Anderson (2) that the "unsaponifiable wax" was optically inactive. We have since found that all fractions corresponding in properties to the "unsaponifiable wax" have a low dextrorotation. At the time of the first investigation the only sodium light available was produced by heating sodium chloride, which gave a rather feeble light, but since that time a sodium lamp has been employed which permits of much closer reading.

Determination of Hydroxyl. Stodola's Method (6)—The methyl ester was used for this determination. Found, OH 1.3.

Determination of Alkoxy—The Zeisel method as modified by Vieböck and Brecher (7) was used. Found, OCH₃ 1.4, 1.3.

It was reported by Anderson (2) that the "unsaponifiable wax" gave a volatile iodide in the Zeisel determination which if calculated as isopropyl iodide would correspond to about 4 per cent of glycerol. However, if the volatile iodide determined by Anderson be calculated as methyl iodide, the value would correspond to 1.34 per cent of methoxyl—a figure in complete agreement with the values obtained in the present investigation.

*

Characterization of the Alkoxy Group. Method of Willstätter and Utzinger (8)—0.4709 gm. and 0.5266 gm. of substance gave 41.3 mg. and 47.6 mg. of tetramethyl ammonium iodide.

The tetramethyl ammonium iodide was recrystallized from alcohol and analyzed for iodine.

$(\text{CH}_3)_4\text{NI}$ (200.92). Calculated, I 63.16; found, I 63.02, 63.17

The results show that the volatile iodide was methyl iodide; hence the alkoxy group is methoxy.

Examination of Iodomycolic Acid—The wax-like non-volatile residues from the experiments mentioned above were combined, washed with water, and precipitated five times from ether solution with methyl alcohol and cooling. The white amorphous powder, which weighed 1.026 gm., melted at 50–51°, and contained 7.19 per cent of iodine.

In another experiment 0.6 and 0.7 gm. of mycolic acid, 3 gm. of phenol, 1 cc. of hydriodic acid, sp. gr. 1.96, and 8 cc. of hydriodic acid, sp. gr. 1.70, were heated to 150° for 2 hours. The methoxy values were 1.29 and 1.33 per cent. The non-volatile residues were combined and purified as described above. The white amorphous product melted at 53.5–55.5° and contained 10.16 per cent of iodine. The equivalent weight determined by titration in ether solution with alcoholic potassium hydroxide was 1570 and the hydroxyl value, determined by Stodola's method (6), was 0.91 per cent.

When mycolic acid was heated with hydriodic acid, sp. gr. 1.96, and phenol, the non-volatile residue after purification as mentioned above gave a white powder, m.p. 41–43°, which contained 19.0 to 19.3 per cent of iodine.

It was reported by Anderson (2) that the residue from the methoxy determination of the "unsaponifiable wax" melted at 46–47° and contained 10.47 per cent of iodine.

The results reported above show that the iodides formed on demethylation of mycolic acid may contain varying amounts of iodine. The reason for this variation probably depends upon the conditions under which the Zeisel determinations are carried out; *i.e.*, concentration of hydriodic acid, time of heating, and temperature. The iodine values found do not agree with any

definite molecular replacement of methoxyl and hydroxyl groups by iodine.

Analysis of Mycolic Acid—The average of five closely agreeing analyses gave the following results. Found, C 81.76, H 13.34.

The simplest formula calculated from these values allowing for one carboxyl, one hydroxyl, and one methoxyl group is $C_{88}H_{172}O_4$ (1292). The values found for molecular weight by titration agree with this formula but the results obtained by the Rast method both for the free acid and its methyl ester are somewhat lower. The value for hydroxyl is also in agreement with the above formula but the percentage of methoxyl is about 1 per cent low, the calculated value for one methoxyl group being 2.4 per cent.

The hydrogen value found is slightly low for a saturated aliphatic compound of this formula but mycolic acid is apparently not unsaturated, because when tested by the Hanus solution it does not absorb any iodine. Neither does it immediately decolorize a chloroform solution of bromine but on longer standing bromine is absorbed apparently by substitution, since hydrobromic acid is liberated. Whether the simplest formula of mycolic acid is $C_{88}H_{172}O_4$ or $C_{88}H_{176}O_4$ cannot be decided from the present data. If the first formula should be correct, then the substance must contain a ring system in its structure. It is also possible that mycolic acid is dibasic, having double the molecular weight indicated above.

Methyl Ester of Mycolic Acid—Attempts to prepare the methyl ester by refluxing mycolic acid with absolute methyl alcohol and dry hydrochloric acid did not yield a fully neutral ester, probably owing to the insolubility of both the acid and the ester in methyl alcohol.

By means of diazomethane in ether solution a neutral ester was easily obtained. For purification the substance was precipitated from ether solution with methyl alcohol. The ester was a white amorphous powder and melted at 43–45°.

A neutral ester was also prepared by refluxing the dried potassium salt of mycolic acid in benzene solution with methyl iodide.

Pyrolysis of Mycolic Acid—In the first study of the "unsaponifiable wax" Anderson (2) found on heating the substance under reduced pressure that normal hexacosanoic acid distilled off at a temperature of about 300° leaving a non-volatile residue. In the

present study on mycolic acid we have confirmed the observation mentioned above and some additional data on the nature of the non-volatile material have been collected.

In the first new experiment 5.0 gm. of mycolic acid were heated in a small distilling flask provided with a wide outlet tube at a pressure of about 0.5 mm. The distillate began to come over at 280°. The heating was continued for 1.5 hours and the temperature of the bath finally reached 350°.

Isolation of Hexacosanoic Acid—The crystalline distillate was dissolved in ether and titrated to phenolphthalein with standard alcoholic potassium hydroxide. The acidity corresponded to 1.18 gm., calculated as hexacosanoic acid. The acid was isolated and recrystallized five times from benzene. Colorless, thin, plate-shaped crystals were obtained which melted at 87–88°.

<i>Analysis</i> — $C_{26}H_{52}O_2$ (396).	Calculated.	C 78.78, H 13.13
	Found.	" 78.69, " 12.94
		" 78.71, " 13.10

Non-Volatile Residue—The residue in the distilling flask was dissolved in ether and the solution on titration with alcoholic potassium hydroxide with phenolphthalein as indicator was found to be completely neutral in reaction. The ethereal solution was cooled and the white precipitate which separated was filtered off. The filtrate was saved. The precipitate was dissolved in ether and again precipitated by cooling. The white powder thus obtained, Fraction A, weighed 0.95 gm. The mother liquor was diluted with methyl alcohol and cooled. The product, Fraction B, which separated was a yellowish wax-like substance weighing 1.01 gm. The first ethereal filtrate mentioned above was diluted with methyl alcohol and cooled. The precipitate, Fraction C, was a yellow salve-like mass which weighed 0.95 gm. The addition of more methyl alcohol to the mother liquor gave Fraction D, 0.10 gm., a white salve-like mass.

The fractions were analyzed and the results are given in Table I.

The first three fractions show such a remarkable similarity in composition and properties that it would seem as if they were a series of isomeric substances which differed only in melting point.

The iodine numbers, if we assume one double bond fully saturated with iodine, indicate a molecular weight of about 900 or a

multiple of this number if two or more double bonds are present. However, a substance having a molecular weight of 900 and having 1 oxygen atom should contain 1.77 per cent of oxygen, whereas the values obtained on analysis show approximately one-half of this amount, and this would indicate that the molecular weight is about 1800. The analyses show that a part of the oxygen is present as methoxyl; tests for hydroxyl were negative. The nature of the rest of the oxygen has not been established.

The neutral fractions described above were produced by rather prolonged heating at a high temperature. In other experiments we have found when mycolic acid is heated under reduced pressure for only a short time, about 20 minutes, to 300–350° that the same quantity of hexacosanoic acid distils over as in the experiment

TABLE I

Properties of Neutral Fractions from Non-Volatile Portion of Mycolic Acid

Fraction	Weight	Melting point	$[\alpha]_D$ in CHCl_3	Methoxyl	Iodine No., Hanus	C	H	O by difference
	gm.	°C.	degrees	per cent		per cent	per cent	per cent
A	0.95	46–57	–1.2	0.49	28.3	85.34	13.76	0.90
B	1.01		–0.5	0.44	26.1	85.50	13.63	0.87
C	0.95		–1.0	0.57	24.0	85.30	13.84	0.86
D	0.10			0.89	25.8	83.89	13.67	2.44

just described, but the neutral non-volatile fractions differ considerably in composition and in the degree of unsaturation from those mentioned above. Further studies will be necessary before definite deductions can be made concerning the nature of the neutral products formed during the pyrolysis of mycolic acid.

SUMMARY

The principal ether-soluble constituent of the wax of the human tubercle bacillus is a saturated hydroxy, methoxy acid of very high molecular weight for which the name mycolic acid is proposed.

The exact formula of mycolic acid has not been established. The substance does not crystallize and is difficult to purify. The average equivalent weight determined by titration was 1284. The simplest formula calculated from the composition is $\text{C}_{88}\text{H}_{172}\text{O}_4$.

or $C_{88}H_{176}O_4$, assuming one hydroxyl and one methoxyl to one carboxyl group.

Mycolic acid melts at $54-56^\circ$ (corrected) and the observed specific rotation was $+1.8^\circ$.

Mycolic acid is acid-fast.

When heated under reduced pressure to a temperature of $300-350^\circ$, mycolic acid is decomposed, yielding a distillate consisting of normal hexacosanoic acid, $C_{26}H_{52}O_2$, and a non-volatile residue which shows no acidic properties.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LV. STUDIES ON THE WAX FRACTIONS OF THE HUMAN TUBERCLE BACILLUS*

BY CHARLOTTE W. WIEGHARD† AND R. J. ANDERSON

(From the Department of Chemistry, Yale University, New Haven)

(Received for publication, August 13, 1938)

In the studies of the lipids of the tubercle bacillus as carried out in this laboratory the bacteria have been exhaustively extracted with a mixture of alcohol and ether and this process has been followed by thorough extraction with chloroform. The alcohol-ether extracts were found to contain phosphatide, acetone-soluble fat, and a small amount of wax-like material. The separation of the alcohol-ether-soluble compounds was accomplished by fractionation of the ethereal solution of the mixed lipids by means of acetone as described in previous papers (1, 2). The crude phosphatide was purified by repeated precipitation from ether solutions with acetone. All of the mother liquors were concentrated by distillation until the ether was removed and the resulting acetone solutions on cooling in ice deposited white amorphous precipitates consisting of wax-like material. The final acetone mother liquors on concentration to dryness left a reddish salve-like material which we have designated acetone-soluble fat.

The composition and cleavage products of the phosphatide (3) and of the acetone-soluble fat (4) have been reported previously.

* The data are taken from the dissertation submitted by Charlotte W. Wieghard to the Faculty of the Graduate School, Yale University, 1937, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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† Holder of the Henry Strong Denison Fellowship at Yale University, 1934-37.

The present report deals with the composition of the wax-like material which precipitated from the acetone mother liquors on cooling.

EXPERIMENTAL

The crude wax used in this investigation had been isolated from the mother liquors in the purification of the phosphatides from human tubercle bacilli, Strains H-37, A-10, A-12, A-13, and A-14, as described by Crowder, Stodola, Pangborn, and Anderson (2). To the combined fractions which weighed 76.5 gm. were added 11.5 gm. of similar material isolated in a previous experiment, thus giving 88.0 gm. of crude wax. In melting point and solubility all of these fractions were similar.

TABLE I
Properties of Wax Fractions

Fraction No.....	I	II	III
Weight, gm.....	32.0	27.5	27.0
Melting point, °C.	45-46	36-38	26-28
$[\alpha]_D$ in CHCl_3 , degrees	-0.8	+19.0	+27.4
Ash, %	0.78	0.75	0.73
Nitrogen, %	0	0.14	0.24
Phosphorus, %	0.32	0.19	0.13
Iodine No.	0	9.1	19.6
Saponification No.	46	122	136

The crude wax was separated into three fractions as follows: First the wax was precipitated five times from ethyl acetate by cooling. The mother liquors on concentration to dryness left a yellowish solid, Fraction III. The substance which had been precipitated from ethyl acetate was next precipitated ten times from ether by the addition of acetone and cooling. The product was a white amorphous powder and was designated Fraction I. The ether-acetone mother liquors on evaporation to dryness left a slightly yellowish solid residue, designated Fraction II. The properties of the wax fractions are given in Table I.

It is evident that the wax fractions are mixtures of several substances. Since it appeared impossible to isolate any definitely

pure substances from such mixtures, the three fractions were saponified separately and the cleavage products were separated as described below.

Saponification of Fraction I—30.0 gm. of the substance were refluxed for 10 hours with 300 cc. of benzene and 300 cc. of methyl alcohol containing 12 gm. of potassium hydroxide. The solution was evaporated to dryness under reduced pressure and the residue was treated with ether and 10 per cent acetic acid, being thus separated into ether-soluble and water-soluble constituents.

Water-Soluble Constituents. Isolation of the Carbohydrate—The aqueous solution which gave a Molisch reaction was neutralized with ammonium hydroxide and concentrated to a small volume *in vacuo*. Addition of neutral lead acetate gave no precipitate but addition of basic lead acetate and ammonia gave a heavy white precipitate. The precipitate was collected, decomposed with hydrogen sulfide, and the filtrate from the latter was concentrated to dryness *in vacuo*. The residue was a thick syrup weighing 0.5 gm. and consisted of crude carbohydrate.

Isolation of Glycerol—The filtrate from the lead precipitate, after being freed from lead with hydrogen sulfide, was acidified with hydrochloric acid and concentrated to dryness *in vacuo*. The residue on extraction with absolute alcohol yielded on purification in the usual manner a thick syrup which weighed 0.4 gm. The presence of glycerol was shown by the preparation of the tribenzoyl derivative by the Schotten-Baumann method (5). The derivative on recrystallization from absolute methyl alcohol gave colorless crystals, m.p. 75–76°.

Examination of the Carbohydrate. Isolation of Inosite Hexacetate—The carbohydrate fraction after being dried *in vacuo* to a hard, glassy mass was refluxed for 6 hours with 35 cc. of acetic anhydride and 1.0 gm. of fused sodium acetate. After the reaction mixture had cooled, it was mixed with water and extracted with chloroform. The chloroform solution was washed free of acid with water and evaporated to dryness *in vacuo*. The residue was dissolved in methyl alcohol, decolorized with norit, and concentrated to a volume of 5 cc. On standing, colorless plate-shaped crystals separated which after recrystallization weighed 0.1 gm. and melted at 213–214°. The melting point is identical

with that of inosite hexaacetate and, since the substance gave a positive Scherer reaction, it is evident that the crystalline product was inosite hexaacetate.

Isolation of Mannose Phenylhydrazone—The mother liquors from the inosite hexaacetate were concentrated and hydrolyzed by refluxing for 10 hours with 5 per cent sulfuric acid. The sulfuric acid was removed quantitatively as barium sulfate, and the filtrate, which contained reducing sugar, after concentration to a volume of about 10 cc. was mixed with 0.2 gm. of phenylhydrazine dissolved in a few drops of alcohol. The crystalline hydrazone which separated was recrystallized from 60 per cent alcohol. The large, colorless, plate-shaped crystals weighed about 5 mg. and melted with decomposition when rapidly heated at 197° and gave no depression when mixed with pure mannose phenylhydrazone. The crystal form and melting point identify the substance as mannose phenylhydrazone.

The mother liquor from the mannose phenylhydrazone gave a trace of osazone when tested in the usual manner, but the amount was too slight to permit of identification.

The water-soluble constituents amounted to 3 per cent of the wax and consisted of glycerol, inosite, and mannose.

Ether-Soluble Constituents. Separation of "Unsaponifiable Wax"—The total ether-soluble constituents, 30 gm., were dissolved in 200 cc. of ether and 300 cc. of cold alcohol were added. The white granular product, after being filtered off, washed with cold alcohol, and dried *in vacuo*, weighed 19.1 gm., corresponding to 63.6 per cent of the wax. This substance, which appeared to be identical with the so called "unsaponifiable wax" previously described (6), was examined as will be described later.

Separation of Fatty Acids As Alcohol-Insoluble Lead Salts—The filtrate from the "unsaponifiable wax" was concentrated until the ether was removed, and an excess of lead acetate dissolved in hot alcohol was added. The heavy white precipitate which separated on cooling was collected, washed with alcohol, and extracted with ether, but no ether-soluble material was obtained. The lead salts were decomposed by shaking with ether and dilute acetic acid, yielding 3.6 gm. of solid crystalline fatty acids which on crystallization from alcohol and from benzene-methyl alcohol yielded thin, colorless, plate-shaped crystals, m.p. 83–84°, molec-

ular weight 399. These properties correspond to those of normal hexacosanoic acid, $C_{26}H_{52}O_2$ (396).

The more soluble portion of the solid acids was combined with similar acids isolated after saponification of Wax Fractions II and III and was purified as will be described later.

Examination of Filtrate from Alcohol-Insoluble Lead Salts. Isolation of Phthiocerol and Fatty Acids—The filtrate from the lead salts was concentrated *in vacuo* to a small volume, diluted with water, and extracted with ether. The ethereal extract was washed with dilute hydrochloric acid and with water, treated with norit, dried over sodium sulfate, filtered, and evaporated to dryness. The residue, 5.6 gm., was an oil when warm and a white solid at room temperature. The substance, dissolved in 20 cc. of warm ethyl acetate, yielded 0.9 gm. of colorless crystals consisting of crude phthiocerol. From the mother liquor were isolated 1.26 gm. of a levorotatory acid and 2.6 gm. of a liquid acid which were combined with similar fractions isolated after the "unsaponifiable wax" had been resaponified as will be described later.

Identification of Phthiocerol—The crystalline substance mentioned above was twice recrystallized from acetone, separating as aggregates of short needles. The crystals melted at 73–74°, solidified at 71–70°, and remelted at 73–74°. $[\alpha]_D^{20}$ in $CHCl_3$ = -4.6° .

Analysis— $C_{26}H_{52}O_2$ (540).

Calculated. C 77.78, H 13.33, OCH₃ 5.74, 2OH 6.29

Found. " 77.83, " 13.35, " 5.22, 5.83, OH 5.80

The properties and composition of this substance are identical with those of the dihydroxy alcohol phthiocerol described by Stodola and Anderson (7).

Another wax fraction isolated in the purification of the phosphatide from Strain H-37 of tubercle bacillus was purified in the manner described above. The substance which weighed 19 gm. had the same properties as Fraction I. On saponification it yielded 14.3 gm. of "unsaponifiable wax," lower fatty acids, and phthiocerol.

Resaponification of "Unsaponifiable Wax"—Since it has been found in this laboratory (8) that the "unsaponifiable wax" prepared as described by Anderson (6) yields on prolonged saponification mycolic acid, certain lower fatty acids, and the alcohol

phthiocerol, it was decided to resaponify the "unsaponifiable wax" fractions mentioned above. The combined fractions, 33.4 gm., were dissolved in 400 cc. of benzene to which were added 15 gm. of potassium hydroxide dissolved in 175 cc. of methyl alcohol, and the solution was refluxed continuously for 80 hours.

The saponification mixture was worked up in the manner already described and the following products were obtained.

	gm.
Alcohol-ether-insoluble fraction, mycolic acid.....	22.7
Fatty acids precipitated by lead acetate.....	7.0
" " not precipitated by lead acetate.....	0.8
Phthiocerol.....	4.0

No water-soluble cleavage products could be found. The results indicate that the "unsaponifiable wax" is a true wax and the fatty acids must be combined with the dihydroxy alcohol phthiocerol.

Examination of Cleavage Products

The phthiocerol fraction was purified by crystallization as described before. The purified substance melted at 73–74°, $[\alpha]_D$ in $\text{CHCl}_3 = -4.6^\circ$, and its composition agreed with the formula $\text{C}_{81}\text{H}_{72}\text{O}_3$.

Fatty Acids Precipitated by Lead Acetate. Isolation of a Levorotatory Acid—The alcohol-insoluble lead soaps dissolved easily in ether. The lead was removed by extracting the ethereal solution with dilute acetic acid. After being isolated in the usual manner, the acid was obtained as a white semicrystalline mass which weighed 7.0 gm., m.p. 37–39°, $[\alpha]_D$ in ether = -9.5° . Tests for hydroxyl and methoxyl were negative. When tested by the Hanus solution, the acid did not absorb any iodine.

The acid was very soluble in organic solvents and could not be obtained in crystalline form. From a slowly cooling alcoholic solution large needle-shaped crystals separated but they dissolved immediately at room temperature.

An attempt was made to fractionate the acid by means of the barium salt. The barium salt, a white amorphous powder, was precipitated from benzene solution by adding methyl alcohol and cooling. The product was thus separated into five fractions whose properties are shown in Table II.

The molecular weights of all of these fractions were practically identical but there were slight differences in optical rotations and in the refractive indices.

Fraction 1, m.p. 37–38°, was analyzed.

$C_{31}H_{62}O_2$. Calculated. C 79.82, H 13.30, mol. wt. 466
 Found. " 79.79, 79.66, H 13.58, 13.35, mol. wt. 466

The general properties of the acid are similar to those reported by Anderson (9) some years ago for the levorotatory acid obtained in the purification of phthioic acid but the levorotation is higher. The values for molecular weight and for carbon and hydrogen are practically identical and agree best with the formula $C_{31}H_{62}O_2$, although in the earlier report (9) the formula $C_{30}H_{60}O_2$

TABLE II
Properties of Levorotatory Acid

Fraction No.	Weight	n_D^{40}	$[\alpha]_D^{20}$ in ether	Mol. wt. by titration
	<i>gm.</i>		<i>degrees</i>	
1	0.9	1.4522	−10.4	466
2	2.0	1.4521	−8.8	466
3	1.0	1.4532	−7.2	464
4	1.3	1.4525	−8.8	469
5	1.3	1.4490	−7.2	462

was suggested because on theoretical grounds a formula with an even number of carbon atoms was preferred. In dealing with non-crystalline substances of high molecular weight such as the acid under investigation, where ordinary criteria of purity are lacking, it is very difficult to determine the exact formula.

Fatty Acids Which Were Not Precipitated by Lead Acetate—The acids which were not precipitated by lead acetate and which were therefore obtained in the separation of phthiocerol were combined, converted into methyl esters, and fractionated through a modified Widmer column. The esters on saponification gave two acids corresponding to tuberculostearic acid, $C_{19}H_{38}O_2$ (10), and the levorotatory acid, $C_{31}H_{62}O_2$; found, molecular weight 467; $[\alpha]_D = -7.0^\circ$.

Alcohol-Ether-Insoluble Fraction, Mycolic Acid—This fraction

corresponded in properties to the hydroxy, methoxy acid to which the name mycolic acid has been assigned. The crude mycolic acid obtained after resaponification as mentioned above melted at 51–53° and in chloroform solution it showed a specific dextro-rotation of +2.1°. The average molecular weight determined by titration was 1251. For purification the substance was treated four times with 700 cc. portions of boiling acetone and the solutions were decanted and allowed to cool. The white precipitates which separated consisted of fine globular particles.

The combined mother liquors on evaporation to dryness left a non-crystalline residue, 0.9 gm., $[\alpha]_D$ in ether = -3.8° , equivalent weight 783. The material was evidently an impure mixture containing some of the levorotatory fatty acid.

TABLE III
Properties of Fractions of Mycolic Acid

Fraction No.	Weight	M. p., uncorrected	$[\alpha]_D$ in CHCl_3	Equivalent weight
	gm.	°C.	degrees	
1	8.2	53–54	+2.22	1303
2	2.5	53–54	+2.25	1301
3	3.6	53–54	+2.68	1297

The main portions of the mycolic acid mentioned above were combined, precipitated five times from acetone, and separated into three main fractions. The properties of the fractions were identical as shown in Table III.

When tested by the Hanus solution the substance did not absorb any iodine.

Analysis—Fraction 1. Found. C 81.43, H 13.26

Methoxyl (Zeisel). Found. OCH_3 1.24

Hydroxyl (Stodola). “ OH 1.99

The analytical values found are very similar to those reported by Stodola, Lesuk, and Anderson (8) for the samples of mycolic acid which they examined, except that the optical rotation and the hydroxyl values are slightly higher.

Pyrolysis of Mycolic Acid—A sample of the purified mycolic acid, when heated under reduced pressure as described by Ander-

son (6, 11) and by Stodola, Lesuk, and Anderson (8), yielded a crystalline distillate consisting of normal hexacosanoic acid, $C_{26}H_{52}O_2$, m.p. 84–85°, molecular weight 394, and a neutral non-volatile residue.

Saponification of Crude Wax Fractions—The Wax Fractions II and III which had been recovered from the mother liquors in the purification of Fraction I as described above were partly unsaturated as shown by the iodine numbers, the melting points were low, and they possessed a relatively high dextrorotation. These fractions, 25.0 gm. of each, were saponified separately as follows: The wax was dissolved in 100 cc. of benzene and 80 cc. of hot 10 per cent alcoholic potassium hydroxide were added. A gummy precipitate separated immediately. After the mixture had stood

TABLE IV
Cleavage Products of Crude Wax Fractions

	Fraction II	Fraction III
	gm.	gm.
Ether-alcohol-insoluble fraction	5.9	2.30
Phthiocerol	1.6	0.80
Fatty acids	16.7	20.46
Carbohydrate.....	1.24	2.43
Glycerol.....	0.50	0.75

for 1 hour, the solution was decanted and refluxed for 1 hour, during which time an additional gummy precipitate separated. The solution was decanted and refluxed for 72 hours in an atmosphere of hydrogen. The cleavage products were separated by the procedure described for Fraction I.

The gummy precipitates which separated in the beginning were combined. This material was soluble in water and consisted of carbohydrate. The carbohydrate was isolated by means of basic lead acetate in the usual manner. The various fractions obtained from the saponification mixture are given in Table IV.

The presence of phthiocerol, carbohydrate, and glycerol would indicate that these fractions consist of complex mixtures of true waxes and carbohydrate esters of fatty acids, as well as glycerides.

Phthiocerol was isolated in crystalline form and identified by melting point and optical rotation.

The glycerol was identified in both cases by means of the tri-benzoyl derivative which after recrystallization from absolute methyl alcohol had the correct melting point, 75-76°.

The carbohydrate fractions contained about 0.5 per cent of phosphorus and on hydrolysis with dilute sulfuric acid gave 42 per cent of reducing sugar calculated as glucose. Pentose color reactions were negative. The carbohydrate from Fraction III was hydrolyzed but the only cleavage product that could be identified was a small amount of inositol. The latter substance after purification crystallized in prismatic needles, gave the reaction of Scherer, and melted at 222-223°. The reducing sugar could not be identified. However, it was not mannose because no insoluble phenyl-hydrazone could be obtained.

Fatty Acids—The fatty acids were combined and separated into solid and liquid acids by means of the lead soap-ether procedure and gave 9.1 gm. of solid acids and 27.0 gm. of liquid acids.

Solid Saturated Fatty Acids—The solid saturated acids from Wax Fractions II and III were combined with the low melting solid acids from Fraction I and were converted into methyl esters. The esters were fractionated through a modified Widmer column at a pressure of about 1 mm. Pure methyl palmitate and methyl stearate were obtained and on saponification the corresponding free acids were isolated and had the correct melting points and molecular weights. In addition a small amount of a higher boiling ester fraction was obtained which on saponification gave an acid with a molecular weight of 311 and which probably consisted of a mixture of stearic and hexacosanoic acids.

Liquid Fatty Acids—The total liquid fatty acids, 27.0 gm., had an average iodine number of about 10, thus indicating that the main portion consisted of liquid saturated acids. The mixed acids were reduced with hydrogen in the presence of platinum oxide and separated into solid reduced acids and liquid saturated fatty acids by repeating the lead salt-ether treatment. The reduced solid acids weighed 3.0 gm. and the liquid saturated acids obtained from the ether-soluble lead salts weighed 24.0 gm. Both fractions were examined as described below.

Liquid Saturated Fatty Acids—The acids were converted into methyl esters and the esters were fractionated through a modified Widmer column.

The more volatile portion of the ester, about 1.0 gm., was optically inactive and on saponification gave a mixture of both liquid and solid acids which could not be separated.

The principal ester fraction had a dextrorotation of $+12.4^\circ$, corresponding in properties to methyl phthioate. The free acid obtained on saponification was a thick oil at room temperature which solidified on cooling and melted at 20° , $[\alpha]_D$ in ether = $+11.0^\circ$, molecular weight 400. The properties of this fraction correspond closely to those of phthioic acid, $C_{26}H_{52}O_2$ (396) (12).

A small quantity of ester residue remained in the flask after the first distillation. The material was a thick oil having a specific rotation of $+9.8^\circ$ and the saponification number corresponded to a molecular weight of 664. No pure substance could be obtained from this residue.

The results recorded above indicate that the principal portion of the liquid saturated fatty acids consisted of phthioic acid.

Examination of Reduced Acids—The solid reduced acids, 3.0 gm., were recrystallized three times from acetone, twice from methyl alcohol, and three times from benzene. Colorless, thin plates were obtained which melted at $80-81^\circ$, molecular weight 399. This acid represents apparently a slightly impure hexacosanoic acid. The mother liquors from the first crystallizations yielded 1.02 gm. of colorless crystals which melted at $59-60^\circ$, molecular weight 301. This material therefore contained some acid of lower molecular weight than hexacosanoic acid.

Ether-Alcohol-Insoluble Substances from Wax Fractions II and III—These fractions which corresponded in solubility to mycolic acid were purified by being precipitated three times from ethyl acetate and five times from acetone by cooling. The products thus obtained were white amorphous powders but they differed in properties from mycolic acid.

The substance from Fraction II was separated into two fractions by treatment with petroleum ether. The more soluble portion, 1.08 gm., melted at $50-55^\circ$, equivalent weight 862; $[\alpha]_D$ in $CHCl_3$ = $+1.4^\circ$. The petroleum ether-insoluble portion after three precipitations from benzene melted at $80-81^\circ$, equivalent weight 420.

The substance from Fraction III after precipitation from

benzene was a semicrystalline powder which melted at 79–81°, equivalent weight 483.

It is evident, therefore, that Fractions II and III do not contain mycolic acid but certain other fatty acids of high molecular weight.

SUMMARY

1. An investigation has been made of the chemical composition of the wax-like substances contained in the alcohol-ether extracts from the human tubercle bacillus which are insoluble in cold acetone.

2. The material can be separated into several fractions which differ decidedly in properties and in composition.

3. On saponification each fraction yields a complex mixture of fatty acids. (a) Saturated normal acids are represented by palmitic, stearic, and hexacosanoic acids. (b) Saturated branched chain acids are tuberculostearic, $C_{19}H_{38}O_2$, phthioic, $C_{26}H_{52}O_2$, and a levorotatory, $C_{31}H_{62}O_2$, acid. (c) The unsaturated fatty acids belong mainly to the C_{26} series and on hydrogenation yield normal hexacosanoic acid. (d) The hydroxy acid, mycolic acid, constitutes the main ether-soluble component of the less soluble wax fraction but is absent from the more soluble fractions.

4. The dihydroxy alcohol phthiocerol, $C_{36}H_{72}O_3$, was present in all of the wax fractions.

5. All of the wax fractions contained both carbohydrates and glycerol.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LVI. THE WAX OF THE BOVINE TUBERCLE BACILLUS*

BY JAMES CASON† AND R. J. ANDERSON

(From the Department of Chemistry, Yale University, New Haven)

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In previous reports from this laboratory the isolation of lipid fractions from the bovine tubercle bacillus has been described (1) and the composition of the phosphatide (2), as well as that of the acetone-soluble fat (3), has been reported.

The wax of the bovine tubercle bacillus constitutes the bulk of the lipids of this organism, and it was found by Anderson and Roberts (1) that the wax amounted to 63.6 per cent of the total lipids and 8.5 per cent of the dried bacteria. Since no information exists concerning the chemical composition of this important fraction of the bovine type of tubercle bacillus, the present work was undertaken in order to supply some information on this subject. The results obtained indicate that the wax is similar in composition to the wax associated with the phosphatide fraction of the human tubercle bacillus (4).

The cleavage products liberated on saponification consisted of a complex phosphorus-containing carbohydrate and glycerol, together with fatty acids and neutral or unsaponifiable matter. The neutral material was free from sterols but contained the dihydroxy monomethoxy alcohol phthiocerol, as has already been

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† Holder of a National Tuberculosis Association Graduate Fellowship at Yale University, 1936-37.

reported (5). The present report deals with the study of the water-soluble constituents, the carbohydrate and glycerol, and with the fatty acids.

EXPERIMENTAL

The material used in this investigation had been isolated by Anderson and Roberts from the bovine tubercle bacillus by extraction with chloroform and designated by them as Bovine Wax III (1). The substance was a yellowish granular powder which was easily soluble in petroleum ether, ether, benzene, and in chloroform, but practically insoluble in acetone, alcohol, and in methyl alcohol.

For purification 176 gm. of crude wax were precipitated five times from ether solution by the addition of methyl alcohol and

TABLE I
Properties of Wax Fractions

	Purified wax	Salve-like wax
Melting point, °C.....	47-54	
P, %.....	0.30	0.18
N (Kjeldahl), %.....	0.16	0.16
Iodine No. (Hanus).....	3.2	20.2
$[\alpha]_D^{20}$ in benzene, degrees.....	+15.58	

cooling. The purified wax thus obtained was a nearly white granular powder which weighed 96.3 gm. The combined mother liquors on evaporation to dryness left a residue, 77.0 gm., of salve-like consistency.

The two fractions were analyzed and the results are given in Table I.

This report deals only with the composition and cleavage products of the purified wax. The salve-like wax was not further examined at this time.

Examination of Purified Wax for Water-Soluble Compounds—A sample of the wax weighing 6.2 gm. was dissolved in 200 cc. of ether and the solution was shaken thoroughly in a separatory funnel with 100 cc. of water. The aqueous layer on evaporation to dryness left a mere trace of residue, while the ethereal solution on evaporation gave the original 6.2 gm. of wax. It is evident,

therefore, that the water-soluble products liberated on saponification of the wax are combined with the fatty acids in the form of water-insoluble compounds.

Saponification of Wax—The purified wax was saponified in two lots by refluxing for 7 hours with 5 per cent alcoholic potassium hydroxide and the cleavage products were isolated as described by Reeves and Anderson (6) in their study of the avian tubercle bacillus wax. The results are given in Table II.

TABLE II
Cleavage Products of Purified Wax

	From 10.72 gm. of wax		From 49.58 gm. of wax	
	gm.	per cent	gm.	per cent
Carbohydrate.....	0.24	2.24	4.48	9.02
Glycerol.....	0.09	0.84	0.66	1.33
"Unsaponifiable wax".....	6.44	60.08	30.26	61.03
Fatty acids.....	2.18	20.33	9.63	19.40
Neutral material.....	0.56	5.22	2.67	5.38

TABLE III
Cleavage Products of "Unsaponifiable Wax"

	gm.
Alcohol-ether-insoluble material.....	31.01
Fatty acids giving alcohol-insoluble lead salts.....	2.25
" " " alcohol-soluble lead salts.....	0.53
Neutral material.....	0.56
Total material recovered.....	34.35

The carbohydrate was isolated in both cases by means of basic lead acetate and ammonia in the usual manner and dehydrated by grinding under absolute alcohol until a white powder was produced. In the first experiment with the smaller amount of wax a portion of carbohydrate was lost.

Further Saponification of "Unsaponifiable Wax" Fraction—Since it has been shown in this laboratory (7) that the material from the human tubercle bacillus designated "unsaponifiable wax" requires prolonged refluxing with alcoholic potassium hydroxide for complete saponification, a second saponification was carried out.

The two fractions of "unsaponifiable wax" mentioned in Table II were combined and refluxed for 72 hours in a solution containing 150 cc. each of benzene and absolute alcohol and 4 gm. of potassium hydroxide. The reaction products were separated according to the procedure described by Stodola, Lesuk, and Anderson (7) and are summarized in Table III.

The various operations during the isolation of the cleavage products after this second saponification thus resulted in a loss of 6.4 per cent.

The ether-soluble products recovered after complete saponification of the purified wax consisted of 51.4 per cent of alcohol-ether-insoluble material which will be designated provisionally as wax acids, 24.2 per cent of fatty acids, and 6.2 per cent of neutral material. The total water-soluble constituents, including carbohydrate and glycerol, amounted to 10.35 per cent.

Examination of Cleavage Products

Identification of Glycerol—The glycerol fraction was identified by means of the tribenzoyl derivative, which latter was prepared by the Einhorn and Hollandt (8) method. After three recrystallizations from methyl alcohol the derivative melted at 75–76° and gave no depression of the melting point when mixed with an authentic sample of glyceryl tribenzoate.

Analysis— $C_{24}H_{18}O_6$ (404). Calculated. C 71.28, H 4.95
Found. " 70.99, " 5.11

The properties and composition identify the substance as glyceryl tribenzoate.

Carbohydrate Fraction—The carbohydrate obtained as mentioned above gave no reduction with Fehling's solution until after it had been hydrolyzed with dilute acid. Pentose and ketose color reactions were negative. The substance on analysis was found to contain 2.20 per cent of phosphorus and only a trace of nitrogen. Sulfur and halogen were absent.

Rotation—0.3005 gm. of substance dissolved in water and diluted to 10 cc. gave in a 1 dm. tube a reading of $+2.03^\circ$; hence $[\alpha]_D^{25} = +67.5^\circ$. There was no mutarotation.

Separation of Organic Phosphoric Acids from Carbohydrate—A portion of the crude carbohydrate weighing 3.36 gm. was dissolved

in 35 cc. of water and the solution which was acid in reaction was neutralized with ammonium hydroxide, after which a solution of lead acetate was added until no further precipitation occurred. The lead salt was collected, washed with water, and decomposed with hydrogen sulfide. After removal of the lead sulfide the filtrate was concentrated to a thick syrup which, on trituration under absolute alcohol, yielded 0.25 gm. of a white powder, designated Fraction A. The alcoholic solution on evaporation to dryness gave 0.19 gm., Fraction B, as a thick syrup.

The filtrate from the lead precipitate was freed of lead in the usual manner and concentrated to a syrup. The latter, by grinding under absolute alcohol, gave 2.66 gm. of a white powder, Fraction C, which consisted of neutral polysaccharide.

Examination of Fractions. *Fraction A*—A concentrated aqueous solution of the substance was neutralized with barium hydroxide, filtered, and mixed with 2 volumes of alcohol. The resulting precipitate, after two reprecipitations in the same manner, gave 60 mg. of a white amorphous barium salt. After the precipitate was dried at 100° *in vacuo*, the following values were obtained on analysis. Found, Ba 26.53, 26.72; P 6.31, 6.29.

The substance gave no reduction with Fehling's solution until after it had been boiled with dilute acid, but it gave a positive Scherer test for inosite. The analytical values would indicate a monophosphoric acid ester of a disaccharide.

$C_{12}H_{21}O_{14}P\text{Ba}$ (557.4). Calculated. Ba 24.65, P 5.56

Fraction B—A portion of the substance when heated with potassium acid sulfate gave a strong reaction for acrolein, thus indicating the presence of glycerol or a glycerol derivative. The substance was converted into the barium salt as described for Fraction A. The product, a white amorphous powder weighing 0.132 gm., was analyzed after it had been dried at 100° *in vacuo*. Found, Ba 42.10, P 10.52, C_8H_7I (Zeisel) 47.14.

It has been found in this laboratory (unpublished data) that synthetic barium glycerophosphate gives in the Zeisel determination the same yield of isopropyl iodide, about 96 per cent of the theory, as does a corresponding amount of pure glycerol. We have also determined that various hexoses and inosite give mere traces of volatile iodide and that barium inosite monophosphate gives no

volatile iodide in the Zeisel determination. The amount of isopropyl iodide found in the above analysis corresponds to about 89 per cent of barium glycerophosphate.

Fraction C—The neutral polysaccharide forming Fraction C could not be obtained in crystalline form, and attempts to fractionate the substance by means of lead acetate were unsuccessful. A portion of the polysaccharide was acetylated but the acetyl derivative did not crystallize. In methyl alcoholic solution the acetyl derivative showed the specific dextrorotation of $+53.7^\circ$. The acetyl value was 46.8 per cent. After saponification the polysaccharide was recovered, but it could not be induced to crystallize.

For purification the polysaccharide was twice precipitated with basic lead acetate and ammonia, the lead salts being decomposed with hydrogen sulfide, and the filtrate from the lead sulfide after concentration to a syrup was dehydrated by grinding under absolute alcohol. The final product thus obtained was a white hygroscopic powder which weighed 1.75 gm. For analysis the substance was dried at 100° *in vacuo* over dehydrite. Found, P 0.72, N 0.18.

Rotation—0.9998 gm. of substance dissolved in water and diluted to 25 cc. gave in a 1 dm. tube a reading of $+3.07^\circ$; hence $[\alpha]_D^{25} = +76.7^\circ$. There was no mutarotation.

Since it was impossible to crystallize the polysaccharide, the substance was hydrolyzed and the cleavage products were determined.

Hydrolysis of Polysaccharide—When the polysaccharide was boiled with dilute sulfuric acid, the maximum amount of reducing sugar determined by the Shaffer-Hartmann method (9) was liberated in 2.5 hours and amounted to 56 per cent calculated as glucose. 1 gm. of the polysaccharide was refluxed with 75 cc. of $N H_2SO_4$ for 2.5 hours, and the cleavage products were examined as described below.

Rotation of Hydrollysate—The hydrolyzed solution was diluted to 100 cc. with water. In a 2 dm. tube the observed rotation was $+0.12^\circ$; hence $[\alpha]_D^{25} = +6.0^\circ$.

Test for Glucosamine—2 cc. of the hydrollysate were tested for glucosamine by the method of Elson and Morgan (10), as modified by Palmer, Smyth, and Meyer (11), but the color obtained in-

dicated only a trace of glucosamine. The nature of the nitrogen contained in the polysaccharide has not been discovered.

Isolation of Cleavage Products. Organic Phosphoric Acids—The sulfuric acid was removed quantitatively with barium hydroxide and after removal of barium sulfate the solution was neutralized with barium hydroxide, concentrated to a volume of about 20 cc., and 25 cc. of alcohol were added which caused a precipitate of a barium salt which weighed only 7 mg. Evidently, therefore, the bulk of the phosphoric acids had been adsorbed on the barium sulfate. The latter on extraction with 1 per cent hydrochloric acid yielded a water-soluble barium salt which on precipitation with alcohol weighed 48 mg. The two fractions of barium salts were dissolved in water, the barium was removed quantitatively as sulfate, and the organic phosphoric acids were precipitated as lead salts with neutral lead acetate. The lead salts, after being decomposed with hydrogen sulfide in the usual manner, yielded a non-crystallizable syrup which weighed 40 mg. On triturating the syrup with absolute alcohol a white powder was produced and on evaporation of the alcoholic solution a syrupy residue was obtained.

Alcohol-Insoluble Phosphoric Acid—The white powder mentioned above, since it could not be crystallized, was dissolved in water and the solution was neutralized with barium hydroxide. On addition of alcohol a white amorphous barium salt was precipitated which weighed 19 mg. For analysis the salt was dried at 100° *in vacuo* over dehydrite.

<i>Analysis</i> — $C_6H_{11}O_6P\text{Ba}$ (395.4).	Calculated.	Ba 34.75, P 7.84
	Found.	" 31.79, " 7.39
		" 31.64, " 7.42

The substance gave a strongly positive Scherer reaction, thus indicating the presence of inosite. The properties and reactions of this substance suggest that it was a slightly impure sample of barium inosite monophosphate (12).

Alcohol-Soluble Phosphoric Acid—The syrupy residue mentioned above was dissolved in water and neutralized with barium hydroxide. The resulting barium salt after three precipitations from aqueous solution with alcohol was a white amorphous powder which weighed 17 mg. The substance gave a positive acrolein

test. For analysis it was dried at 100° *in vacuo* over dehydrite. Found, Ba 39.35, P 7.73; C_8H_7I (Zeisel) 21.09.

The results of the analyses indicate that the substance contained about 38 per cent of barium glycerophosphate.

Isolation of Mannose and Inosite—The hydrolysate from which the barium salt had been removed by precipitation with alcohol was quantitatively freed of barium with sulfuric acid and concentrated *in vacuo* to a volume of 10 cc. Mannose and inosite were isolated from this solution in the manner described in an earlier paper (13).

The yield of mannose phenylhydrazone was 0.76 gm., corresponding to 50.6 per cent of mannose. The melting point and mixed melting point, as well as crystallographic measurements of the recrystallized hydrazone, identified the substance as mannose phenylhydrazone.

The yield of inosite by direct crystallization was 0.084 gm. The substance gave the Scherer reaction and melted at 224° . When mixed with inactive inosite, there was no depression of the melting point.

The mother liquor from the inosite crystallization on evaporation to dryness left a syrupy residue which weighed 0.48 gm. The syrup had a specific rotation of $+3.3^{\circ}$ and contained reducing sugar, but no definite sugar derivative could be isolated from this material.

Wax Acids—The alcohol-ether-insoluble material recorded in Table III was precipitated five times from ether solution by addition of an equal volume of alcohol and cooling. The product thus obtained was a nearly white amorphous powder which weighed 23.97 gm. and which will be designated Wax Acid I.

The mother liquor on concentration until the ether was removed gave 7.04 gm. of a slightly straw-colored amorphous powder, designated Wax Acid II.

Wax Acid I. Isolation of Bovine Mycolic Acid—The crude Wax Acid I was easily soluble in cold benzene and in chloroform, also in warm ether and in hot ethyl acetate, but almost insoluble in acetone and in methyl, ethyl, or isopropyl alcohol. For purification the substance was precipitated six times from ether solution by cooling and separated into six fractions. These fractions varied in melting point from 67° to 51° and the equivalent

weights determined by titration ranged from 1755 to 1213 for the top and bottom fractions, respectively, thus showing that the substance was a mixture. Those fractions which possessed the highest equivalent weight evidently contained some neutral material, and it was found that the latter could be eliminated by precipitating the acid as the sodium salt from ether solution. The several fractions were dissolved in ether, 125 cc. per gm. of crude acid, and titrated with alcoholic sodium hydroxide, phenolphthalein being used as indicator. The sodium salts which

TABLE IV
Analyses of Fractions from Wax Acid I

Nature of determination	Acid fractions		
	Top	Middle	Bottom
Weight, gm.....	0.6	5.9	1.6
M. p., °C.....	71-75	56-58	52-54
Equivalent weight by titration....	1203	1219	1180
$[\alpha]_D$ in CHCl_3 , degrees	+1.5	+2.7	+1.7
Methoxyl, %.....	0.63	0.92	1.31
Carbon, %.....	81.87	82.10	81.53
Hydrogen, %	13.38	13.60	13.32
	Ester fractions		
M. p., °C.....	55-60	44-46	43-45
Hydroxyl, %.....	1.26	1.42	1.37
Methoxyl, total, %...	2.97	3.34	3.61
Carbon, %.....	81.97	81.90	81.52
Hydrogen, %.....	13.46	13.59	13.34

The methoxyl was determined by the micro-Zeisel method (14) and the hydroxyl value according to Stodola (15).

separated as amorphous precipitates were collected and the free acids were liberated by shaking the precipitates with ether and dilute hydrochloric acid. The acids were again precipitated from ether solution by addition of methyl alcohol and were obtained as white amorphous powders. In this manner eight fractions were obtained, total weight 18.75 gm. The melting points varied from 71° to 52° but the equivalent weight was about 1200 for every fraction, varying from 1180 to 1219.

The results indicate that all the fractions were mixtures and it

appears unlikely that any pure compound can be separated by ordinary methods from such mixtures of non-crystalline material. However, in order to gain some insight into the type of compounds with which we were dealing, the top, middle, and bottom fractions were analyzed and the results are given in Table IV.

The methyl esters were prepared by methylation with diazomethane and were precipitated as white amorphous powders from ether solution by addition of methyl alcohol.

Since the melting point is the highest in the top fraction, the rotation at a maximum in the middle fraction, and the methoxyl content is highest in the bottom fraction, it would seem as if at least three different substances must be present. All of the fractions, however, gave approximately the same hydroxyl value, corresponding to one OH group per carboxyl in an acid having a molecular weight of about 1200. However, the actual molecular magnitude cannot be determined from the present data.

Bovine Mycolic Acid—The properties of the middle fraction of Wax Acid I are very similar to those of mycolic acid (7) isolated from the human tubercle bacillus, Strain H-37; hence we propose to designate this substance by the name bovine mycolic acid. When mycolic acid is heated under reduced pressure to a temperature of 250–300°, it cracks and *n*-hexacosanoic acid distills off. We found that bovine mycolic acid behaves in a similar manner. When a portion of the bovine mycolic acid weighing 1.93 gm. was heated as described by Anderson (16), a crystalline distillate was obtained which weighed 0.42 gm. After six recrystallizations from benzene-methyl alcohol 0.25 gm. of snow-white crystalline plates was obtained, m.p. 87–88°, equivalent weight 399.

Analysis— $C_{26}H_{52}O_2$ (396). Calculated. C 78.78, H 13.13
Found. " 78.79, " 13.17

The data show that the acid was *n*-hexacosanoic acid.

Wax Acid II—This fraction was also a mixture from which no pure substance could be isolated. Separation by means of the ether-insoluble sodium salt resulted in a mixture of acids. The principal fraction had the following properties, m.p. 74–77°, equivalent weight 632, methoxyl (Zeisel) 0.67.

Analysis—Found, C 80.23, H 13.33.

The methyl ester was prepared as described before; m.p. 49–53°, hydroxyl 0.94, methoxyl, total, 4.48.

Analysis—Found, C 80.10, H 13.32.

An adequate study and characterization of the wax acids must await the development of new methods for the separation and purification of acids of such high molecular weight.

Fatty Acids of Lower Molecular Weight—The fatty acids isolated after the saponifications as recorded in Tables II and III were separated by the lead salt-ether procedure into 6.78 gm. of solid saturated acids and 7.62 gm. of acids giving ether-soluble lead salts.

Acids from Ether-Soluble Lead Salts—The crude acids formed a semisolid mass and had an iodine number of 5. The mixture was subjected to catalytic reduction with hydrogen and platinum oxide, after which the lead salt-ether separation was repeated.

The solid reduced acid thus isolated weighed 0.46 gm. From methyl alcohol it separated as a white amorphous powder having an indefinite melting point 55–90°, equivalent weight 316. This mixture was not further investigated.

The acids recovered from the ether-soluble lead salts, 6.5 gm., were converted into methyl esters. The esters were fractionated and refractionated in a high vacuum through a modified Widmer column, yielding two principal portions, Fraction I and Fraction II.

Fraction I—The ester, 1.44 gm., distilled as a colorless oil at 112–114° and 0.006 mm. pressure, melted at about 0°, was optically inactive, $n_D^{25} = 1.4436$, $d_4^{25} = 0.8620$, and the saponification value corresponded to a molecular weight of 301.

<i>Analysis</i> — $C_{19}H_{33}O_2$ (298).	Calculated.	C 76.51, H 12.75
	Found.	" 76.10, " 12.74
		" 76.07, " 12.77

The free acid, 1.21 gm., isolated after saponification was a colorless crystalline mass, m.p. 29–30°.

<i>Analysis</i> — $C_{18}H_{33}O_2$ (284).	Calculated.	C 76.05, H 12.67
	Found.	" 75.93, " 12.73
	Mol. wt.,	284.5

The 2,4,6-tribromoanilide of the acid was prepared according to Robertson (17), and the product was recrystallized from methyl alcohol until the melting point remained constant at 96–96.5°. The substance crystallized as fluffy, snow-white needles.

Analysis— $C_{24}H_{48}ONBr_3$ (596). Calculated. C 48.32, H 6.43
Found. " 48.35, " 6.44

The analytical data indicate that this acid is an analogue of tuberculostearic acid (18) and an isomer of stearic acid with a branched chain.

Fraction II—The ester, 1.86 gm., distilled at 172–175° and 0.003 mm. pressure, m.p. 22–24°, $[\alpha]_D^{20} = -5.3^\circ$, $n_D^{25} = 1.4519$, $d_4^{25} = 0.8562$. The acid, after saponification of the ester, was obtained as a wax-like solid, m.p. 33–34°, $[\alpha]_D^{20} = -3.98^\circ$, equivalent weight 430.

The properties of this acid resemble those of the levorotatory acid isolated from the human tubercle bacillus (19), but it was apparently a mixture from which no pure tribromoanilide could be obtained.

The ester residue from the first distillation also yielded a mixture of levorotatory acids but no pure substance could be isolated.

The results indicate that the acids from the ether-soluble lead salts consisted of (a) higher unsaturated acids which could not be identified, (b) an optically inactive saturated branched chain C_{18} acid, and (c) certain levorotatory acids of high molecular weight which could not be adequately separated or identified.

Solid Saturated Fatty Acids—The acids isolated from the ether-insoluble lead salts were completely saturated because they absorbed no iodine when tested by the Hanus solution. The methyl esters were prepared and systematically fractionated through a modified Widmer column at a pressure of about 4 mm. into six fractions.

Fraction I. Isolation of Palmitic Acid—The ester was a colorless oil which crystallized at room temperature, melted at 27–28°, and weighed 2.29 gm. Saponification yielded a white crystalline acid which after three crystallizations from methyl alcohol melted at 62–63° and gave no depression when mixed with pure palmitic acid.

Analysis— $C_{16}H_{32}O_2$ (256). Calculated. C 75.00, H 12.50
Found. " 74.94, " 12.36
Mol. wt., 252

The properties and composition identify this acid as palmitic acid.

Fractions II to IV were apparently intermediate fractions and were not examined.

Fraction V, which melted at 36–37°, was thought to be methyl stearate, but the acid obtained on saponification melted at 65–66° and depressed the melting point of stearic acid about 9°; hence stearic acid if present at all must be a very minor constituent.

Fraction VI. Isolation of a Tetracosanoic Acid—The crystalline ester melted at 39–42° and the acid obtained on saponification was recrystallized from acetone until the melting point remained constant at 76–77°. The crystal form was quite different from that of the ordinary fatty acids; bulky diamond-shaped crystals separated from acetone and boat-shaped needles were obtained from methyl alcohol.

Analysis— $C_{24}H_{48}O_2$ (368). Calculated. C 78.26, H 13.04

Found. " 78.20, " 13.12

Mol. wt., 368, 367

The analytical data are in agreement with the calculated composition of tetracosanoic acid, but the low melting point and peculiar crystal form suggest that the substance is probably a branched chain acid.

Unsaponifiable or Neutral Material—From the neutral material liberated on saponification of the wax, as recorded in Tables II and III, was isolated the dihydroxy monomethoxy alcohol phthiocerol, $C_{35}H_{72}O_3$, m. p. 73–74°, as described in a previous report (5). In addition to phthiocerol the neutral material contains a second non-crystalline substance which we have not investigated.

SUMMARY

The results of our investigation of the purified chloroform-soluble wax from the bovine type of the tubercle bacillus may be summarized as follows:

1. The composition of the wax is in general similar to the wax contained in the alcohol-ether extract of the human tubercle bacillus and which is obtained from the mother liquors in the purification of the phosphatide fraction.

2. The following types of compounds were obtained on saponification of the wax: (a) water-soluble constituents, glycerol and carbohydrate, (b) the ether-alcohol-insoluble Wax Acids I and

II, (c) ether-alcohol-soluble fatty acids, and (d) neutral or unsaponifiable matter.

3. The carbohydrate is a complex mixture of organic phosphoric acids and a phosphorus-containing neutral polysaccharide. The neutral polysaccharide on hydrolysis yields (a) organic phosphoric acids, among which inosite monophosphoric acid is apparently present, (b) mannose, (c) inosite, and (d) an unidentified reducing sugar.

4. The ether-alcohol-insoluble Wax Acid I represents a mixture of optically active acids with an average molecular weight of about 1200, containing hydroxyl and methoxyl groups. The chief component is bovine mycolic acid which is very similar in its properties to mycolic acid obtained from the wax of the human tubercle bacillus. Bovine mycolic acid, when heated under reduced pressure to 250–300°, cracks and *n*-hexacosanoic acid distills off.

Wax Acid II represents a mixture of optically active acids having an average molecular weight of about 630 and containing hydroxyl and methoxyl groups.

5. The ether-alcohol-soluble fatty acids consisted of (1) solid saturated fatty acids which on fractionation yielded (a) palmitic acid and (b) a tetracosanoic acid of unknown constitution; (2) a small quantity of unsaturated fatty acids of high molecular weight but of unknown composition; (3) a new saturated optically inactive branched chain acid, $C_{18}H_{36}O_2$, isomeric with stearic acid; (4) a mixture of levorotatory acids having an average molecular weight of about 430 which could not be separated or identified.

6. The only ordinary fatty acid found among the ether-soluble constituents of the bovine tubercle bacillus wax was palmitic acid.

7. The neutral or unsaponifiable matter contained a crystalline dihydroxy monomethoxy alcohol, $C_{38}H_{72}O_3$, identical with phthiocerol, which has previously been found only in the waxes from the human tubercle bacillus. About one-half of the neutral material was a non-crystalline substance of unknown composition.

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APPLICATION OF THE SCHOENHEIMER-SPERRY METHOD TO THE DETERMINATION OF CHO- LESTEROL AND CHOLESTEROL ESTERS IN TISSUES*

BY STUART STURGES AND ARTHUR KNUDSON

*(From the Department of Biochemistry, Medical Department of Union
University, Albany Medical College, Albany)*

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In the course of studies planned on cholesterol metabolism, it was felt necessary to develop a microtechnique for cholesterol that would be suitable for analysis in tissues.

It has been pointed out that a direct Liebermann-Burchard reaction method is dependent upon too many variables, some of which cannot be regulated, to be accurate for colorimetric work. There is also the possibility of other chromogenic substances interfering with the color reading. Because of these factors, the basic method of Schoenheimer and Sperry (1) was chosen. It provides for a finer separation of cholesterol from other chromogenic substances by means of a digitonide precipitation and an indirect Liebermann-Burchard reaction for color development, the variables of which can be regulated.

Colorimetry was carried out with the use of a colorimeter equipped with microcells and a light filter. The filter is necessary because digitonin by this method produces a slight color which is avoided by reading between 610 and 630 $m\mu$, the maximum absorption for digitonin. The method of extraction is that of a simple reflux, completeness of which is absolutely essential for accurate results.

* A report of this work was presented before the Division of Biological Chemistry at the meeting of the American Chemical Society at Rochester, September 6-10, 1937. The work was aided by a grant from the Committee on Scientific Research, American Medical Association.

Procedure

Reagents—The materials are the same as in the original method of Schoenheimer and Sperry with the exception that a 0.3 per cent alcoholic solution of digitonin in 90 per cent alcohol was used instead of a water solution.

The following apparatus was used: (1) 10 cc. graduated cylinders with glass stoppers; (2) 12 cc. centrifuge tubes with stirring rods. Each tube had a tip of not more than 2 to 3 mm., which facilitated the removal of liquids;¹ (3) a colorimeter with a filter



FIG. 1. Apparatus as set up for extraction of tissue cholesterol

(Wratten No. 71A) and microcells, as suggested by Fitz (2); (4) a water bath enclosed so that twenty tubes could be kept in the dark; (5) 1 quart preserving jars with rubber gaskets; (6) an electric fan; (7) a finger reflux, as illustrated in Fig. 1. This consists of pieces of glass tubing small enough to be inserted in the necks of the 10 cc. volumetric cylinders. The length of these tubes is about 3 inches. They are sealed at one end and on the other are placed pieces of rubber tubing about an inch long. Each of the pieces of rubber has a small hole pierced in its side through which is passed a second tube of 6 to 8 mm. stock, long enough to reach

¹ These tubes were prepared specially for us of Pyrex glass by the Corning Glass Works.

the bottom of the condenser tube and drawn to a long, fine tip of about 1.5 mm. This second tube acts as the inlet tube. Three of these condensers are set in series. On the outside of each is also placed a small rubber disk that acts as an apron and prevents dust and moisture from getting into the flasks.

Preparation of Tissue—The tissue was first bared and cleaned. With skin tissue, the hair was first removed by means of fine hair clippers and a razor. As much as was possible of the fatty layer was then removed, so that the remaining portion consisted largely of epithelium and corium. In the case of organ tissue, the fat, if present, was removed, leaving just the organ for use. Tissues were then handled in one of three ways.

The first two methods required shredding of tissue with scissors until a very fine subdivision was obtained. The tissue was weighed in a 10 cc. volumetric cylinder which had been previously dried at 100° and weighed. In the first method, absolute alcohol was added in sufficient amount to cover the tissue and after standing for half an hour, it was placed in an oven at 100° for 1 hour. It was then removed to a vacuum desiccator where it was placed under reduced pressure overnight. The next day, the tubes were weighed and the material extracted. The second method was to extract the tissue directly and determine the dry weight of the tissue by taking a separate aliquot sample. The sample was weighed on a dried and weighed glass, covered with absolute alcohol, placed in an oven at 100° for an hour, removed to a desiccator and placed under reduced pressure overnight, and then weighed.

In the third method, which applies only to skin, the tissue was dried at a low temperature, as suggested by Thaysen (3). The tissue was cleaned and placed in an oven at 32–36° in such a manner that a slow current of air would pass over it. When it was thoroughly dried, the tissue was cut in small pieces, placed in the oven for a few more hours, weighed in a volumetric cylinder of known weight, and extracted.

The amount of tissue used for determinations varied with the amount of cholesterol present. For skin, about 200 mg. of wet tissue were found to be sufficient. The smallest amount of skin tissue used was a sample of about 50 mg.

Extraction—To each of the cylinders containing tissue, 3 to 4 cc.

of acetone-alcohol are added. These are placed on a sand bath with a finger reflux (Fig. 1) placed in the neck of each and allowed to reflux for 3 hours. The cylinders are then raised and cooled until ebullition ceases. The reflux tubes are raised and washed with several small portions of acetone-alcohol by means of a pipette with a rubber bulb. The cylinders are then removed from the bath, cooled, and made up to a volume of 10 cc. The contents are mixed thoroughly and the extract is ready for determination. If, however, a cloudy extract is obtained, it should be filtered as for blood, but usually the solution is clear enough to use directly.

Determination

Precipitation of Free Cholesterol—An aliquot of 1 or 2 cc. is pipetted into a centrifuge tube containing a glass stirring rod. 3 drops of 5 per cent HCl are added and then 1 cc. of alcoholic digitonin. The solutions are mixed and placed in a glass jar overnight.

Precipitation of Total Cholesterol—To 1 cc. of extract in a centrifuge tube with a glass rod, 1 drop of the KOH solution is added. The solutions are stirred and placed in a tightly sealed jar at 37° for 2 hours. The tube is then removed, cooled, and neutralized to phenolphthalein with 5 per cent HCl. Then sufficient water is added to dissolve the salt present. To this is added 1 cc. of digitonin solution. These are stirred and placed in a jar at room temperature overnight. From here, the steps of centrifugation and washing, drying, and color development for both the free and total cholesterol are the same as in the original method (1).

A Klett microcolorimeter with a Wratten filter (No. 71A) was used for the determination of color intensities. At the side of the colorimeter was placed an electric fan, so that the fumes of the acetic anhydride would not interfere with the measurement. This also helped cool the cups while readings were being made, thus reducing the possibility of error due to heat, which changes the color.

DISCUSSION

The technique of the Schoenheimer-Sperry method has been shown for blood to produce results that are comparable with the macromethod of Windaus (4). The latter is believed to be accu-

rate to within 2 per cent. In order to check the accuracy of our microcolorimetric method on tissues, a comparison of the total and free cholesterol was made with the gravimetric method of Fex (5) as modified by Sperry (4). Our first difficulty arose in attempting to get a representative sample for the microextraction method. The skin from the face, back, and abdomen of several rats was used and treated as described above in the third method for preparation of tissue. After drying, it was chopped into very small pieces and mixed thoroughly. A portion was then taken for the micromethod. For the macrogravimetric method, about 15 gm. of tissue were used. The results of these comparisons are given in Table I.

TABLE I

Comparison of Colorimetric and Gravimetric Methods for Cholesterol in Skin

The figures represent mg. per 100 gm. of tissue.

Rat skin	Total cholesterol		Free cholesterol	
	Microcolorimetric method	Macrogravimetric method	Microcolorimetric method	Macrogravimetric method
A	205	204	93	90
B*	278	269	100	104
C*	276	267		

* Rat Skins B and C are taken from one composite sample of skin from four rats, which was divided into two parts.

The agreement between the two methods is very good and indicates that our method of extraction is quite complete and that the values found by our procedure are within 3 to 4 per cent of that obtained by the gravimetric method.

A further check upon the completeness of extraction was carried out by collecting about 30 gm. of extracted tissue. This was washed three times with alcohol-acetone solution to remove any of the adhering cholesterol-containing liquid. It was then put into solution with 2 per cent NaOH and extracted by the method of Fex. The resulting extract contained no cholesterol.

In the course of development of our method, the precipitation of cholesterol was studied. Schoenheimer and Dam (6) had reported that for alcohol solutions 20 per cent of water is necessary for complete precipitation. However, in Table II it will be noted

that in an alcohol-acetone tissue extract containing hydrochloric acid solution complete precipitation of cholesterol is obtained with variation of the water content from 6 to 20 per cent.

It has been reported that acid tends to inhibit coupling of digitonin and cholesterol. However, we have found practically the opposite, as less water is required to bring about complete precipitation if only a slight amount of acid is present. With distilled water alone, variable results were produced. At times precipitation did not occur and at other times a very greasy precipitate that was not complete would be formed. Checks were obtained with 2 drops of 0.1 N H_2SO_4 and 1 drop of a saturated solution of

TABLE II
Variation in Water Content in Precipitation of Cholesterol from Tissue Extract

Each tube contained 1 cc. of tissue extract and 1.5 cc. of alcoholic digitonin.

Tube No.	5 per cent HCl	Total water	Water	Total cholesterol
	cc.	cc.	per cent	mg. per 100 gm.
1	0.076	0.151	5.9	222
2	0.152	0.227	8.6	222
3	0.228	0.303	11.1	220
4	0.304	0.379	13.5	220
5	0.380	0.455	15.7	225
6	0.456	0.531	17.9	222
7	0.532	0.607	21.0	221

potassium dichromate in H_2SO_4 , instead of a solution of hydrochloric acid. The total water present was 6 per cent, giving some indication that acids aid, rather than hinder, the reaction.

Owing to the separation of other materials when a water solution of digitonin was added, an alcoholic solution was used. Sufficient water was later added in the form of a 5 per cent solution of hydrochloric acid for precipitation. Two different brands of digitonin² were used for precipitation of cholesterol, and, as far as our results were concerned, they were equally efficient.

In Table III are given a few representative results of analysis

² The two digitonins tested were from Merck and Hoffman-La Roche.

of several tissues of the normal rat to which this method has been applied.

The rats from which these tissues were taken were about 1 year old. Several determinations were made on the skin from the face, back, and abdomen. The total cholesterol averages about the same from each of these places, but the free cholesterol varies and it is highest in skin from the face, averaging about 60 per cent. The skin from the back is lowest in free cholesterol, averaging

TABLE III
Cholesterol in Tissues of Rats

Tissue	Solids	Cholesterol		Free in total cholesterol
		Total	Free	
	<i>per cent</i>	<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>	<i>per cent</i>
Skin from face	36.1	225	126	56
	31.5	209	116	55
	37.9	215	145	67
" " back	42.1	212	99	45
	40.0	241	67	27
	39.8	184	57	31
" " abdomen	43.4	252	109	43
	39.2	202	105	52
	24.9	352	324	90
Kidney	24.2	450	406	93
	25.3	195	175	89
Liver	28.2	189	150	84
	28.5	150	139	92
	29.0	202	158	73

about 35 per cent, and the skin from the abdomen is between these two, averaging about 47 per cent.

In the kidney tissues, the total cholesterol is high and 90 per cent is in the free state. The liver tissue has only about half as much cholesterol as the kidney tissue and from 73 to 92 per cent is in the free state.

SUMMARY

1. A microcolorimetric method for the determination of tissue cholesterol has been devised, utilizing the basic method of Schoenheimer and Sperry.

2. The microcolorimetric tissue procedure checks with the macrogravimetric method.

3. The water content for complete precipitation need not be 20 per cent, as suggested in the procedure of Schoenheimer and Sperry.

We would like to express our appreciation to Dr. Schoenheimer and Dr. Sperry for their interest in the problem and their most valuable suggestions.

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THE DETERMINATION OF ETHYL ALCOHOL IN BLOOD AND URINE WITH THE PHOTOELECTRIC COLORIMETER

BY JOHN G. GIBSON, 2ND, AND HARRY BLOTNER

(From the Medical Clinic of the Peter Bent Brigham Hospital and the Department of Medicine, Harvard Medical School, Boston)

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In the method described by Nicloux (1) the concentration of alcohol in biological fluids is determined by measuring the amount of potassium dichromate in solution in sulfuric acid reduced by the alcohol present, the reaction involved being $3C_2H_5OH + 2K_2Cr_2O_7 + 8H_2SO_4 = 3CH_3COOH + 2K_2SO_4 + 2Cr_2(SO_4)_3 + 11H_2O$. The excess of potassium dichromate over that entering into the reaction is determined by titration with potassium permanganate after the addition of ferrous ammonium sulfate.

In the modification of this method herein described, the concentration of alcohol is measured by a direct determination with the photoelectric colorimeter of Evelyn (2) of the diminution in color of the acid dichromate solution, due to reduction by the alcohol. This procedure does away with the preparation of the quantitative ferrous ammonium sulfate and the unstable potassium permanganate solutions and with the titration.

Potassium dichromate in sulfuric acid solution has a stable color which conforms to the Beer-Lambert law, its absorption spectrum being as shown in Fig. 1, Curve 1. In the reduction of potassium dichromate in acid solution by ethyl alcohol a green color develops, the intensity of which increases with the amount of dichromate reduced. Thus, as shown in Fig. 1, Curve 5, when enough alcohol has been added to reduce completely all the dichromate present, the solution instead of being colorless has a definite green tinge.

The development of this secondary color results in a progressive shift in the peak of the transmission band of the dichromate solu-

tion towards the violet as the amount of dichromate reduced increases (see Fig. 1, Curves 1 to 5).

The filter employed is composed of a yellow glass 3 mm. thick and a blue glass 2 mm. thick. The transmission curves of the

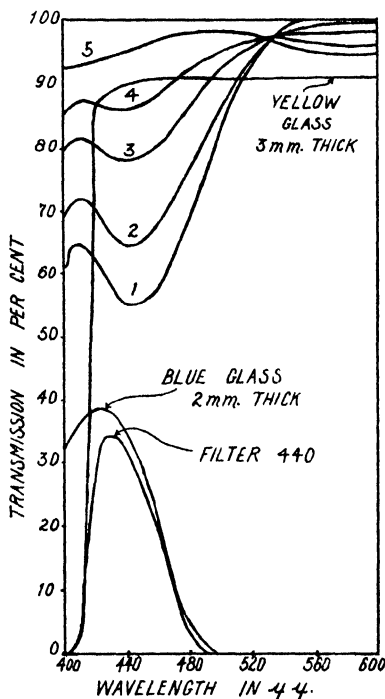


FIG. 1. Spectrophotometric transmission curves of component yellow and blue glasses and composite transmission of Filter 440, and of acid potassium dichromate solutions to which ethyl alcohol has been added. Curve 1, 0.213 gm. per cent $K_2Cr_2O_7$ in 5 N H_2SO_4 ; Curves 2 to 4, same, to which alcohol in concentrations of 31.2, 62.4, and 78.0 mg. per cent have been added. In Curve 5 the $K_2Cr_2O_7$ is completely reduced. There has been a progressive shift towards the violet of the transmission peak of the yellow color and an increase in absorption about 590 $\mu\mu$.

component glasses and of the composite Filter 440¹ are shown in Fig. 1. The use of this fairly wide spectral band permits of ob-

¹ Stock filter supplied with the photoelectric colorimeter by the Rubicon Company, Philadelphia.

taining transmission values bearing a logarithmic relationship to the intensity of the combined yellow and green color and hence to the concentration of alcohol, in spite of the shift in absorption peak (see Fig. 2).

Method

Reagents—

1. Aqueous solution of potassium dichromate, 3.1943 gm. per liter.
2. 10 per cent aqueous solution of sodium tungstate.
3. $\frac{2}{3}$ N sulfuric acid.
4. 18 N sulfuric acid.

*Procedure—*A modification of the Fleming and Stotz method (3), with the Folin blood filtrate, is used. 2 cc. of oxalated whole blood² are laked in 14 cc. of water and 2 cc. of the sodium tungstate solution added and the solution shaken. The addition of 2 cc. of 18 N sulfuric acid precipitates both serum protein and hemoglobin and the mixture is either filtered or centrifuged until the supernatant fluid is clear. 10 cc. of the filtrate are placed in a 100 cc. round bottomed flask and about 5 to 6 cc. distilled off through a glass tube leading into a 25 cc. ground glass-stoppered measuring cylinder containing 5 cc. of 18 N sulfuric acid and 2 cc. of the potassium dichromate solution. The volume after distillation should be close to 15 cc. to obtain an approximately 6 N solution of H_2SO_4 . The cylinder is cooled in an ice bath during distillation and the open end of the tube should reach nearly to the bottom of the cylinder. After distillation, the ground glass stopper is sealed in place with a drop of concentrated sulfuric acid and the contents of the cylinder heated in a water bath at a temperature of about 75–80° for 20 minutes, then cooled to room temperature, diluted to exactly 20 cc., mixed, and decanted into a standard colorimeter tube.

The amount of urine employed is 10 cc. when the concentration of alcohol is low, as before, and 1 cc. made up to a volume of 10 cc. with distilled water when high, as after the ingestion of alcohol. The urine is treated in the same way as the blood fil-

² The skin was sterilized for venipuncture with a 1:1000 bichloride of mercury solution.

trate. The method, however, is not specific for alcohol, because volatile reducing substances such as acetone give the test.

Colorimetry

A blank is prepared by the addition of 5 cc. of 18 N sulfuric acid to 15 cc. of water in a colorimeter tube. The 10 cc. aperture of the colorimeter is used, and with the blank in place the galvanometer is adjusted to 100 with Filter 440. The reading obtained after removal of the blank tube from the instrument (the center setting) is kept constant for readings on all test samples. With each set of samples a reading is made on a tube containing 2 cc. of the 3.1943 gm. per liter potassium dichromate, 5 cc. of 18 N H_2SO_4 , and 12 cc. of distilled water. This tube corresponds to a 0 mg. per cent standard.

The concentration of alcohol in the original material is obtained by the formula

$$X = \frac{100V(L_1 - L_2)}{AK} \quad (1)$$

where X is the number of mg. of alcohol in 100 cc. of blood or urine; V (in cc.) is the final volume of the solution placed in the colorimeter tube (20 cc.); A (in cc.) is the volume of original material used in the test (1 cc. of blood and 10 cc. of urine); and L_1 and L_2 are the negative logarithms of the galvanometer readings of the 0 mg. per cent standard and of the sample tube respectively. K is the calibration constant, the numerical value of which has been determined as 7.50 with the type of instrument used in this investigation.

A table may be constructed from the calibration chart from which the concentration of alcohol in mg. per cent for any galvanometer reading may be read directly.

Calibration

Calibration consists in the determination of the values of the constant K by measurements on solutions containing known concentrations of alcohol.

A calibration curve was made from the transmission values³

³ Corrected for linear aberration of the galvanometer according to the calibration table supplied with each instrument.

of a series of alcohol solutions of known concentration and is shown in Fig. 2. The alcohol was anhydrous and the concentration of alcohol in the series of standards is as given in Table I. For each standard 1 cc. of the alcohol solution was added to 2 cc. of 3.1943 gm. per liter potassium dichromate, 5 cc. of 18 N H_2SO_4 , and 7 cc. of distilled water in a graduated measuring cylinder, making the volume 15 cc. This mixture was heated in a stoppered cylinder in a water bath at 80° for 20 minutes, cooled to room temperature, diluted to 20 cc. with distilled water, decanted into a standard colorimeter tube (2), and read in the photometer with Filter 440, the 10 cc. aperture being used, against an appropriate blank tube prepared as described above. The correspond-

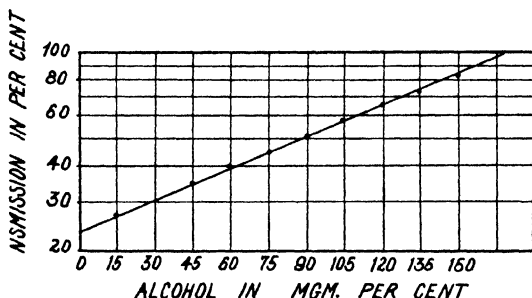


FIG. 2. Calibration curve. Logarithmic relationship of transmission values (G) obtained with Filter 440 for acid $\text{K}_2\text{Cr}_2\text{O}_7$ solutions and concentration of ethyl alcohol.

ing values of K were then calculated from Equation 1. From the results given in Table I, $K = 7.5$ when $A = 1$ and $V = 20$. Identical values for K were obtained in a series of standards in which 1 cc. of the alcohol standard was diluted to 10 cc. and subjected to distillation into the acid dichromate solution, as described above under "Procedure."

When this method is adopted, it is only necessary to check the accuracy, for each photoelectric colorimeter, of the K factor, as given in Table I, by means of one or two carefully prepared alcohol standards made as described above. In principle this method is adaptable to any photoelectric colorimeter, and, theoretically, to any two-cup colorimeter in which the Gillespie principle is employed.

TABLE I
Determination of Colorimetric Constant

Alcohol	<i>G</i>	<i>L</i> ₁	<i>L</i> ₁ - <i>L</i> ₂	<i>K</i>
<i>mg. per cent</i>				
0	23°	0.3617*		
15	27°	0.4314	0.0697	9.30
30	29°	0.4624	0.1007	6.72
45	34 ¹	0.5346	0.1729	7.68
60	38 ¹	0.5827	0.2210	7.37
75	44°	0.6435	0.2818	7.52
90	49 ¹	0.6902	0.3285	7.31
105	55 ²	0.7443	0.3826	7.30
120	64 ²	0.8096	0.4479	7.47
135	75 ¹	0.8765	0.5148	7.62
150	84 ²	0.9269	0.5652	7.53
Average.....				7.50

G is the galvanometer reading, expressed to nearest quarter of a scale division.

* *L*₁, or 2 - log *G* of sample of K₂Cr₂O₇ containing no alcohol.

TABLE II
Amount of Alcohol Recovered by Titration and Photoelectric Colorimeter Method from Distillation of Alcohol Solutions of Known Concentration
The values are given in mg. per cent.

Known concentration of alcohol	Amount recovered	
	Photoelectric colorimeter	Titration
98	95	91
78	75	74
62	58	59
47	43	44
39	36	35
31	29	30
23	21	24
16	15	13
8	6	
4	4	
2	1-0	1-0
1	1-0	

It seemed of practical value to compare the results obtained with the titration and photoelectric methods in a series of alcohol solutions of known strength. These are shown in Table II, and are much the same with both methods. However, in repeating the tests a number of times on the known solutions, the results with the photoelectric method were more consistent and reliable than with the titration method. A small amount of alcohol such as 1 or 2 mg. was not recovered at all times. It is common experience with a distillation method to recover slightly less alcohol than is present in the test sample.

TABLE III

Comparison of Alcohol Concentrations in Blood and Urine after Ingestion of Alcohol As Determined by Titration and Photoelectric Colorimetric Method

The values are given in mg. per cent.

Blood		Urine	
Titration	Photoelectric colorimeter	Titration	Photoelectric colorimeter
11	10	15	11
17	18	18	20
24	27	20	23
35	34	30	32
40	43	39	44
49	53	44	42
50	45	55	57
55	61	67	74
62	68	85	94

In Table III are shown the results obtained by both methods on blood and urine samples of nine patients taken after the ingestion of alcohol. The slight difference in results obtained is of no clinical significance.

Normal Blood and Urine Alcohol

Specimens of blood of 52 normal fasting or non-fasting individuals, known to have taken no alcohol for some time previous to the test, were tested for alcohol by the method described. In thirty-three of these people, simultaneous samples of urine were obtained. In twenty-nine cases, the urine alcohol concentration

ranged from 0.4 to 2.4 mg. per cent and in four cases there was no alcohol in the urine. In thirty cases, the blood contained no alcohol or less than 1 mg. per cent, and of the urines tested in this group the alcohol concentration varied from 0 to 0.9 mg. per cent. In twenty-two cases, the blood alcohol concentration ranged from 1.0 to 6.5 mg. per cent, the usual amount being 2 to 4 mg. Of the urines tested in this group the alcohol concentrations varied from 0 to 2.4 mg. per cent. There was no apparent relationship between the presence or absence of alcohol in the blood and in the urine in this series of cases.

Investigators using other methods have found the alcohol content of normal blood to vary somewhat. Kridelka and Bohet (4) found no volatile reducing substance in normal blood. Friedemann and Klaas (5) obtained approximately 0.4 mg. per cent for the alcohol content of normal blood and urine. Harger and Goss (6) found from 0 to 0.027 mg. per cent of alcohol in normal blood, and from 0.06 to 0.185 mg. per cent of alcohol in normal urine. Miles (7) and Gettler *et al.* (8) found higher values for normal blood, ranging from 1.7 to 4.0 mg. per cent. In the opinion of Taylor (9) and Gettler and coworkers (8) the reducing substance found is actually ethyl alcohol, although it is possible that acetone may also reduce the dichromate. It is evident that the blood of normals may contain traces of ethyl alcohol in the absence of recent ingestion of alcohol.

SUMMARY

1. A photoelectric colorimetric method for determining the concentration of ethyl alcohol in blood and urine based on the direct measurement of the diminution in the color of potassium dichromate resulting from the reduction by alcohol is described.

2. This procedure eliminates the preparation of two quantitative solutions, one being unstable, and substitutes objective colorimetry for titration.

3. With this method described the alcohol content of normal blood varies from 0 to 6.5 mg. per cent, and of normal urine from 0 to 2.4 mg. per cent.

Miss Y. L. Dondos and Miss Evelyn Berstein gave valued technical assistance.

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A MICROMETHOD FOR THE DETERMINATION OF IRON IN BLOOD

BY ROSALIE BREUER AND WALTER E. MILITZER

(From the Avery Laboratory of Chemistry, University of Nebraska, Lincoln)

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In studying the accepted clinical methods for the determination of iron in whole blood, we encountered difficulty in obtaining check results with the Wong (1) method and also with other methods. This is not unusual and has been reported by others in the literature and in private communications (2). Our experiences in trying to get a practical method that would give check results run parallel with those of Jackson (3) on a variety of biological materials. Since he has discussed the factors involved rather fully, our results will only be mentioned here. Dry ashing methods on a microscale never gave consistent values and always led to low recoveries. Of the wet ashing methods, only the use of perchloric acid gave a satisfactory oxidation of the organic matter. It has, however, the disadvantage of being dangerous for routine laboratory or clinical use (4); the procedures are long, involved, and unsuited for microdeterminations.

We have found that acid permanganate can be used very satisfactorily for the digestion of small samples of blood. The oxidation results in a clear solution free from interfering substances. The excess permanganate can be destroyed with a drop of hydrogen peroxide and the iron determined by the thiocyanate method after stabilization of the color.

Fading of the ferric thiocyanate is caused by the evolution of hydrogen sulfide from the decomposition of the thiocyanate ion. This reduces the ferric ion to the colorless ferrous ion which gives no color with thiocyanate. The decomposition is hastened by light and by a strong acid medium. The color can be stabilized for several hours even in strong acid by placing the solutions in the dark and by adding an oxidizing agent to oxidize the hydrogen

sulfide. The stabilizers that have been mentioned in the literature are nitric acid and potassium persulfate (1). Of the oxidizing agents we have tried in the stabilization, potassium persulfate is superior, since it has only a small tendency to oxidize the thiocyanate to free sulfur. Hydrogen peroxide is also satisfactory but has a greater tendency in this direction.

The following method is a micromethod. Former methods have required macro samples of blood. The procedure is based on 0.1 cc. of blood, but it can be reduced to 0.05 cc. without difficulty. Although a volumetric flask is recommended for best results, an accurately calibrated Pyrex test-tube will serve just as well.

Procedure

Reagents—

1. Iron-free distilled water.
2. Iron-free concentrated sulfuric acid.
3. 2 per cent solution of potassium permanganate.
4. 3 per cent solution of hydrogen peroxide.
5. Saturated solution of potassium persulfate, 7 gm. per 100 cc. of water.
6. Potassium thiocyanate solution, 14.6 gm. dissolved and diluted to 50 cc. with water; made up fresh about every 3 weeks.
7. Standard iron solution. Weigh out sufficient ferrous ammonium sulfate of known iron content to give 0.1 mg. per cc. of iron when diluted to 1 liter. This generally amounts to 0.7 gm. Dissolve in 50 cc. of distilled water and add 50 cc. of 10 per cent iron-free concentrated sulfuric acid. Warm and add 0.1 N potassium permanganate until oxidation is complete. Transfer accurately to a liter volumetric flask and dilute to the mark. This is the primary standard. For the secondary standard dilute exactly 10 cc. of the above to 100 cc.

1 cc. of iron-free distilled water is placed in a 10 cc. glass-stoppered volumetric flask. 0.1 cc. of blood is pipetted into this by means of a 0.1 cc. micropipette. Lowering of the pipette to the mark is greatly aided by holding a piece of filter paper over the tip. The contents of the flask are drawn into the pipette up to the mark several times to insure removal of the blood. The last traces of blood are rinsed from the pipette by drawing dis-

tilled water into the pipette up to the mark and expelling into the flask. 1 cc. of concentrated sulfuric acid is added and the flask rotated to insure solution of the laked blood.

2 per cent potassium permanganate solution is now added drop by drop from a burette, with shaking after each addition. The total amount generally required is about 2 cc. The first cc. may be added fairly rapidly but as the oxidation proceeds to completion the additions should be made in 2 and 3 drop portions. During the addition the temperature of the solution is kept about 60° by an occasional whirling over a small microburner. When the permanganate color persists for 5 minutes, the digestion is completed. The excess is destroyed by a drop of 3 per cent hydrogen peroxide. If manganese dioxide is formed during the oxidation, it may, likewise, be removed with hydrogen peroxide before the next addition of permanganate.

The flask is cooled and 0.5 cc. of saturated potassium persulfate solution is added, after which water is added up to the 10 cc. mark. Then 1 cc. of potassium thiocyanate solution is added. The contents of the flask are thoroughly shaken after being stoppered, and a portion is filtered directly into a colorimeter cup through iron-free filter paper. It is preferable to prepare each sample separately and measure its color at once, rather than prepare a number of samples together.

Comparison is made with a standard solution set at 20.0 prepared as follows: 5 cc. of the secondary standard are pipetted into a 10 cc. volumetric flask. 1 cc. of concentrated sulfuric acid is added; after mixing, the flask is cooled, 0.5 cc. of saturated persulfate is added, and then the whole diluted to the mark with water. 1 cc. of potassium thiocyanate solution is added, followed by a thorough mixing. The solution is placed directly in a light-protected colorimeter cup and allowed to remain there during the comparisons.

Since the standard contains 0.05 mg. of iron, the calculation in mg. per 100 cc. of blood follows from the formula $(20/R) \times 0.05 \times (100/0.1)$, in which R denotes the colorimeter reading.

Some of the results are given in Table I.

Although the values in Table I indicate a close agreement, the precision of the method cannot be greater than that of the color-

imeter. This varies with the observer but generally amounts to two-tenths or three-tenths of a division on the scale. This represents about 0.6 mg. of iron on the basis of 100 cc. of blood.

TABLE I
Determination of Iron in Blood

Sample	Colorimeter reading, average	Fe per 100 cc.
	<i>mm.</i>	<i>mg.</i>
A1	19.0	52.6
A2	19.0	52.6
B1	21.8	45.9
B2	21.9	45.7
C1	18.3	54.6
C2	18.3	54.6
D1	16.8	59.5
D2	16.7	59.9
E1	17.3	57.8
E2	17.3	57.8
F1	20.9	47.8
F2	20.9	47.8
G1	17.5	57.2
G2	17.3	57.8
H1	16.8	59.5
H2	16.6	60.2

TABLE II
Comparison of Hemoglobin Content by Newcomer Method and Iron Method

Sample	Hb calculated from Fe	Hb by Newcomer method
	<i>per cent</i>	<i>per cent</i>
A	15.7	15.8
B	13.7	13.8
C	16.3	16.7
D	17.9	18.1
E	17.3	17.4
F	14.3	13.8
G	17.2	17.3
H	17.9	18.1

We attempted to check the figures by the old and new Wong methods, and the Kennedy method; but these never showed agreement within themselves, and, hence, were discarded. For

purposes of comparison we determined the hemoglobin content of the bloods by the Newcomer method (5). In Table II the hemoglobin calculated from the iron content as compared with the hemoglobin determined by the Newcomer method is given in per cent. Iron content may be converted to per cent hemoglobin by dividing the mg. per 100 cc. by 3.35.

The values check within the experimental error of the Newcomer method, which is subject to an error in color matching to the extent of 0.2 to 0.4 per cent in hemoglobin.

In order to check the accuracy of the method in relation to the absolute iron content small quantities of iron were added to digested samples. The additions were made on bloods of iron

TABLE III
Recoveries of Iron Added to Samples of Blood

Fe in 0.1 cc. sample	Additions	Fe determined	Recovered
mg.	mg.	mg.	per cent
0.0448	0.01	0.0544	96
	0.01	0.0542	94
	0.02	0.0633	92.5
	0.02	0.0637	94.5
0.0455	0.01	0.0553	98
	0.01	0.0549	94
	0.02	0.0649	97
	0.02	0.0646	95.5

content in the lower region of normal blood. The recoveries are given in Table III.

The recoveries fall within the experimental error of the method and within the accuracy demanded in rapid clinical use. For bloods extremely low in iron content a standard within range of the sample should be used in place of the usual standard. This avoids colorimeter errors and errors in color depths of the ferric thiocyanate which are somewhat dependent on the concentration of iron (6).

SUMMARY

A method for the determination of iron in micro samples of blood has been given. Check results are easily obtainable with

the method which is more rapid and direct than the existing macromethods. Hemoglobin calculated from the iron checks within experimental error with the per cent hemoglobin as determined by the Newcomer method. Recoveries of added iron, although not 100 per cent, are within experimental error of colorimetry and well within the accuracy demanded of a clinical method.

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A METHOD FOR THE QUANTITATIVE ESTIMATION OF GLUCURONIC ACID AND CONJUGATED GLUCURONIDES

By G. B. MAUGHAN,* KENNETH A. EVELYN,† AND J. S. L. BROWNE

(From the Department of Medicine, McGill University Clinic, Royal Victoria Hospital, Montreal, Canada)

(Received for publication, August 2, 1938)

The study of the metabolism and excretion of glucuronic acid has assumed added importance in recent years because of the discovery that the sex hormones are excreted in the urine of the human as glucuronide complexes. Cohen and Marrian (1), by the isolation and identification of estriol glucuronide in human pregnancy urine, have shown that estrin is excreted, at least in part, as a complex of glucuronic acid. Venning and Browne (2) have shown that the sodium pregnanediol glucuronidate found in urine of women is an excretion product of progesterone. Peterson and his coworkers (3) have isolated a physiologically inactive form of androgenic substance from human urine which is probably a complex of glucuronic acid.

The methods at present used for the quantitative estimation of glucuronic acid are all prolonged and tedious procedures. None of them is completely accurate. The methods most widely used in recent years are modifications of the one described by Quick (4) for menthol glucuronic acid. Since, in this procedure, the glucuronide is extracted from urine by ether, and since the sex hormone glucuronides at least are not soluble in ether, the inaccuracy of the method for the estimation of the total glucuronide content of urine is self-evident.

In 1908 Tollens (5) described a method for the qualitative

* Hiram N. Vineberg Fellow in Obstetrics and Gynecology, McGill University.

† Aided by a grant from The Banting Research Foundation, Toronto, Canada.

detection of glucuronic acid. This method is based on the formation of a blue-violet compound by the reaction of the glucuronide with naphthoresorcinol on being heated with hydrochloric acid. The following year Tollens (6) modified this method in an attempt to make it quantitative. Mandel and Neuberg (7) challenged the specificity of Tollens' test, finding many other compounds which would give a blue-violet color. None of these, however, occurs in urine in sufficient quantity to invalidate the specificity of the test when carried out under our conditions. In our experience mucic acid is the only compound, other than glucuronic acid, which gives the typical color, and this occurs only when high concentrations are used.

It was thought that by making use of the increased sensitivity afforded by the use of the photoelectric colorimeter (8) the Tollens test could be made quantitative. It was found, however, that the technique for the development of the colored compound had to be changed in several important details.

It was observed that naphthoresorcinol in absolute alcoholic solution gave relatively poor color development and that decreasing the concentration of alcohol increased the amount of color developed. For this reason an aqueous solution of naphthoresorcinol has been used. The reagent is prepared by weighing out the naphthoresorcinol (Eastman) into a flask and adding enough distilled water to make a 0.2 per cent solution. The flask is stoppered, kept at a temperature of about 38° for 24 hours, and shaken at intervals. The solution is then filtered and kept in the dark in a refrigerator at 3-5°. The reagent will keep for about a month, but should be tested from time to time against a standard solution of glucuronide. During this time the amount of color produced from a given amount of glucuronide remains constant within the limits of reproducibility of the method (± 2 per cent). There is, however, a progressive increase in the amount of color developed in the blank tube, and the reagent should be discarded when the galvanometer reading after removal of the blank tube is greater than 80. By means of this change in technique, the sensitivity of the Tollens test has been increased from 50 to 100 times. Urines containing normal amounts of glucuronic acid must be diluted from 10 to 20 times to give readings within the range of accuracy of the colorimeter because

solutions containing more than 40 micrograms of glucuronic acid per cc. give galvanometer readings too low for accurate estimation.

For constant results the ether used for extraction must be of good quality (we have used Merck's Reagent), washed immediately before use with a 1 per cent solution of ferrous sulfate to remove peroxides which destroy the color, and then washed once with distilled water and dried with a few crystals of sodium sulfate in the bottom of the flask.

Method

In a colorimeter test-tube¹ are mixed 2 cc. of the solution of glucuronic acid or glucuronide to be tested, 2 cc. of the 0.2 per cent naphthoresorcinol solution, and 2 cc. of concentrated hydrochloric acid. The tube is heated in a boiling water bath for exactly 30 minutes and is then placed in an ice bath for 5 to 10 minutes. 2 cc. of 94 per cent ethyl alcohol and 15 cc. of the washed ether are added to the tube. (It has been found that the addition of the alcohol facilitates the extraction of the colored compound by the ether.) The tube is corked immediately after the addition of the ether (the use of a rubber stopper is inadvisable), shaken vigorously end to end for 20 to 30 seconds, and the mixture is allowed to separate into layers. For the colorimetric reading a filter transmitting light in the region of 565 m μ is used (see Fig. 1). In order to make the reading on the supernatant fluid without transferring the volatile liquid to a second tube, the standard tube holder of the instrument is replaced by one which allows the light beam to pass through the solution at the appropriate level.¹ The galvanometer is adjusted to 100 with a tube prepared and carried through the steps of the procedure in exactly the same fashion as described above, except that 2 cc. of distilled water are used instead of the glucuronic acid solution; then a reading is made on the sample tube. From this reading the amount of glucuronic acid in the original 2 cc. aliquot of diluted urine can be calculated from the formula, $C = (2 - \log G)/K \times 100$, where C is micrograms of glucuronic acid, G is the galvanometer reading, and K is a constant whose value is ap-

¹ The colorimeter, filters, tubes, and special tube holder may be obtained from the Rubicon Company, 29 North Sixth Street, Philadelphia.

proximately 0.75 as determined by a series of 200 readings on standard solutions of sodium pregnanediol glucuronidate. However, to guard against changes in chromogenic power with the aging of the naphthoresorcinol solution the value of K should be checked occasionally by preparing with each lot of unknowns a standard tube containing 2 cc. of a glucuronic acid standard

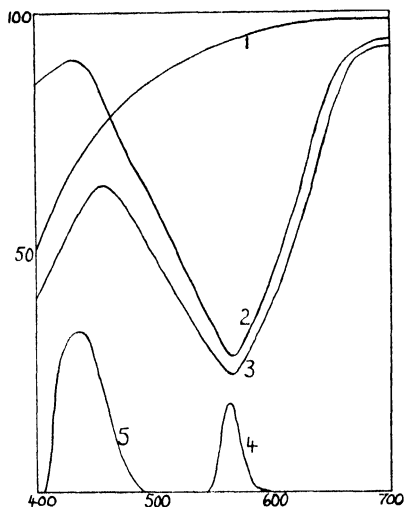


FIG. 1. Spectrophotometric curves of purple and brown colors and of the filters used in their determination. Abscissa, wave-length in $m\mu$; ordinate, percentage of light transmission; Curve 1, brown contaminant present in urine; Curve 2, purple color developed from pure glucuronic acid; Curve 3, mixed purple and brown developed from normal urine; Curve 4, Filter 565; Curve 5, Filter 440. The curves of the various colors are merely approximations for illustrative purposes only, and are not presented as accurate spectrophotometric data.

solution (we have used sodium pregnanediol glucuronidate for this purpose) containing 20 micrograms per cc. ($C = 40$ micrograms).

DISCUSSION

In the course of color development from urine a brown color develops which, in the vicinity of $565 m\mu$ where optimum light absorption for the blue-violet color occurs, causes little interference (see Fig. 1). In a series of 50 different urines this source

TABLE I
Recovery of Glucuronide Added to Urine

Amount added to sample	Theoretical amount in sample	Amount recovered	Per cent error
<i>micrograms</i>	<i>micrograms</i>	<i>micrograms</i>	
0		598	
36.2	634.2	630	-0.66
72.4	670.4	670	-0.06
181	779	788	+1.15
362	960	956	-0.42

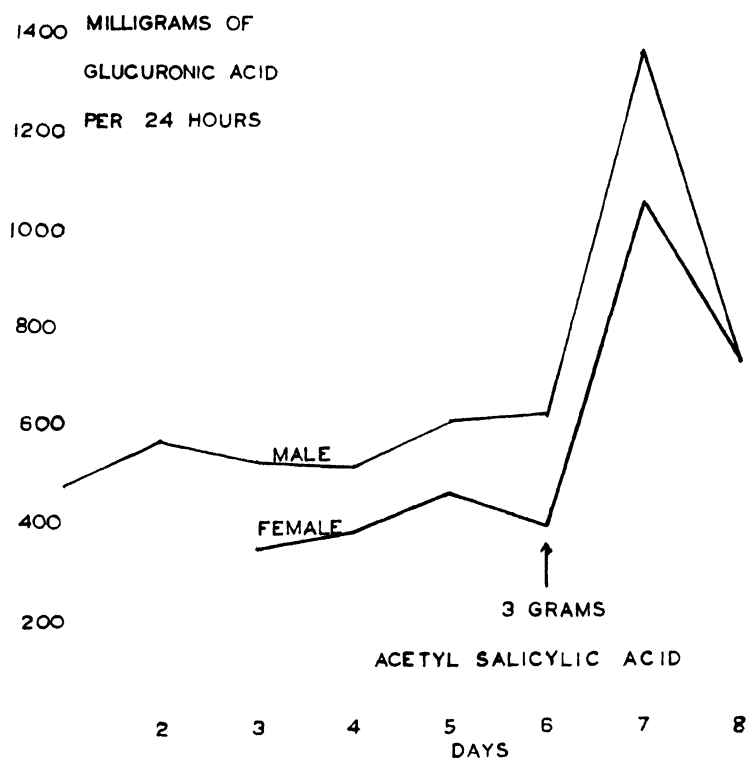


FIG. 2. The excretion of glucuronic acid in mg. per 24 hours over several consecutive days in the urine of two normal individuals. At the point indicated by the arrow 3 gm. of acetylsalicylic acid were given orally.

of error was found to be fairly constant at about 20 to 40 mg. of glucuronic acid per 24 hour specimen. In the average urine this represents only about 6 per cent of the total glucuronic acid content. For most purposes an error of this magnitude may be neglected, but if greater accuracy is required a correction can be made by making a second measurement on each sample with a filter (No. 440) which is more sensitive to the brown compound and less sensitive to the purple. The principle of this method has been described elsewhere (9).

The glucuronic acid of added sodium pregnanediol glucuronide can be recovered accurately from urine as illustrated in Table I. The glucuronic acid content of estriol glucuronide can also be determined by this method. Sample values for glucuronic acid excretion over 24 hour periods in the urine of two normal individuals are shown in Fig. 2.

It is readily seen that the daily excretion varies considerably. The feeding of 3 gm. of acetylsalicylic acid is seen to cause a prompt marked increase in the amount of glucuronic acid excreted.

SUMMARY

1. A simple and accurate photoelectric method for the quantitative estimation of glucuronic acid in human urine is described.

The authors wish to acknowledge the valuable technical assistance of Mrs. E. V. Harkness and the helpful suggestions of Dr. O. F. Denstedt of the Department of Biochemistry.

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STUDIES ON THE RELATIVE EFFICIENCY OF VITAMIN D FROM SEVERAL SOURCES

I. INFLUENCE OF VITAMIN D OF DIFFERENT ORIGINS ON BONE ASH AND BODY WEIGHT OF THE CHICKEN

BY JOHN T. CORRELL AND E. C. WISE

(From the Research Laboratories, The Upjohn Company, Kalamazoo)

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Within the last few years the dual assay for vitamin D, with both rats and chickens, has become a popular method for distinguishing between the several forms of the antirachitic vitamin as they are found in numerous fish liver oils and other sources. The ash content of either the femur or the tibia of the chicken has been used as the basis of interpreting the responses obtained.

By the use of this technique Massengale and Nussmeier (1) and other workers were able to demonstrate that the vitamin D of irradiated ergosterol and that of cod liver oil were two separate entities.

Further information on the vitamin D problem was contributed by Waddell (2), who reported that upon the rat unit basis the vitamin D of irradiated cholesterol was more effective for the chick, as measured by the bone ash procedure, than the vitamin D of irradiated ergosterol. Furthermore, the irradiated cholesterol compared favorably in that respect with the vitamin D of cod liver oil. This demonstration was an incentive for the study of cholesterol as a precursor of vitamin D. The interest in this relationship culminated in 1936 in the isolation by Windaus, Schenck, and von Werder (3) of an antirachitic substance, vitamin D₂, as the crystalline dinitrobenzoate and allophanate, from the irradiation product of 7-dehydrocholesterol. In the same year Brockmann (4) reported the isolation, as the crystalline dinitrobenzoate, of the same substance from tuna liver oil, and Grab (5) reported a similarity in the antirachitic efficacy, for the chick, of vitamin D₂ from the two sources.

These results were followed in 1937 by the isolation by Brockmann (6) of vitamin D₃ from halibut liver oil. This was the only antirachitic substance the presence of which was detected in this oil. Also, Brockmann and Busse (7), working with liver oil of the blue-fin tuna from Japanese waters, concluded that the vitamin D of that species is identical with the antirachitic vitamin of the tuna and halibut liver oils previously reported.

In the meantime additional information had accumulated relative to the comparative physiological response of the rat and the chick to vitamin D from various sources. Rygh (8) reported in 1935 that the vitamin D of seventeen species of fish was equally effective for the chicken, and Dols (9) found that the vitamin D of tuna liver oil was as effective as that of cod liver oil upon the rat unit basis.

Thus, although it was recognized that the vitamin D of fish liver oils varied considerably in concentration, in general it was thought to consist only of one substance until Bills, Massengale, and Imboden (10) reported that the vitamin D of blue-fin tuna liver oil is only about one-sixth as active as the antirachitic factor of cod liver oil when measured on chickens. Also, Haman and Steenbock (11) and Black and Sassaman (12) indicated the inefficiency of tuna liver oil as compared with cod liver oil under similar conditions.

This confusion has been admirably explained by Bills, Massengale, Imboden, and Hall (13). They point out in an extensive study of fish liver oils that tuna oils vary, according to species, from those which are about equal to those which are only about one-sixth as effective as cod liver oil, rat unit for rat unit, as demonstrated by the bone ash content of chickens. Evidence of this nature indicates that the vitamin D of fish liver oils may consist of more than one antirachitic substance.

The present studies were undertaken to determine the relative efficiency of several different fish liver oils as compared with cod liver oil by means of the rat-chicken technique. We were anxious also to ascertain whether other criteria besides chicken bone ash might further establish the differences or similarities in the physiological response of the various forms of vitamin D.

It is the purpose of this paper to report briefly our findings on approximately 150 chicken assays, the results of which confirm

the conclusions of Bills and others (13) concerning tuna liver oil. Also, we submit data on groups of chicks which have been raised on vitamin D supplements of varying origin for a considerably longer period of time than is necessary for the ordinary chicken assay. These latter investigations demonstrate the growth-promoting effect of vitamin D and the differences in growth response of the chicken to vitamin D from several sources.

EXPERIMENTAL

All oils were first assayed in our own laboratories for vitamin D with the rat by the standard U.S.P. XI method. For the most part our samples of fish liver oils were taken from relatively large lots and are representative of the oils of commerce and, therefore, do not represent oils from strictly selected species.

For the chicken tests, single comb white Leghorn chicks with a uniform nutritional background were used in the prophylactic type of experiment. The birds were started on assay when 1 day old and the bone ash experiments terminated on the 21st day. The growth investigations were prolonged for 8 weeks. In both studies the chicks were weighed and freshly supplemented diets mixed each week. In all cases the basal diet employed was that of Hart, Kline, and Keenan (14), modified to contain 57 parts of maize and 2 parts of dried yeast.

The oils to be assayed were incorporated into the basal ration in quantities calculated to furnish definite dosages of vitamin D in each 100 gm. of diet. When necessary, maize oil was added to the vitamin-bearing adjuvant to bring the total oil supplement of the feed to 1 per cent. The negative control diets were supplemented with 1 per cent of maize oil.

Data which we have accumulated from a large number of chicken assays indicate that the bone ash response to a fixed dose of vitamin D from cod liver oil varies from one time to the next, even under the most uniform conditions. Thus the establishment of a master response curve for vitamin D from cod liver oil, to which the results obtained on feeding vitamin D from other sources could be referred, seemed impractical for this prophylactic chick technique. The most satisfactory method appeared to be the feeding of two or three parallel groups of chicks graded doses of a reference cod liver oil, and the use of these data as a standard

of comparison. One group received no vitamin D and served as a negative control. Griem (15) has arrived at similar conclusions as to the use of the prophylactic chick test for vitamin D.

Twelve to fifteen chicks were started in each group; the sex was fortuitous. Ash determinations were made on pooled left tibiae, alcohol- and ether-extracted and moisture-free, from ten individuals.

TABLE I

Average Bone Ash from Groups of Ten Chickens, Supplemented with Different Sources of Vitamin D

Vitamin D per 100 gm. ration	Tibia ash from groups fed					
	Cod liver oil	Tuna Liver Oil 1	Tuna Liver Oil 2	Tuna Liver Oil 3	Albacore liver oil	Irradiated ergosterol
<i>international units</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0	34.1					
6	39.7	37.2		38.5		
12	42.7	38.0		38.8		
24	45.5	41.8	38.7	42.3		
50		46.1	46.8			
0	34.2*					
9	37.8*	38.8†	38.9†	36.0		
18	44.4*	39.7†	39.8†	39.7		
27	46.7*	43.4†	43.3†	43.4		
0	34.7					
18	46.5		41.1		43.7	35.0
50			47.2		45.2	36.0
80			46.8		47.6	
100						39.5

* Average of three consecutive assays.

† Average of two consecutive assays.

Results

Table I indicates the relative ineffectiveness of several different tuna liver oils as compared with cod liver oil in the calcification of bone. In general, they all appear to be from 40 to 60 per cent as efficient, at the levels fed, as was cod liver oil. Irradiated ergosterol fed at a 100 unit level gave an ash response equivalent to that which would be produced by about 10 international units of vitamin D from cod liver oil. These data substantiate the

reports in the literature which indicate the comparative inefficiency of some tuna liver oils and of irradiated ergosterol.

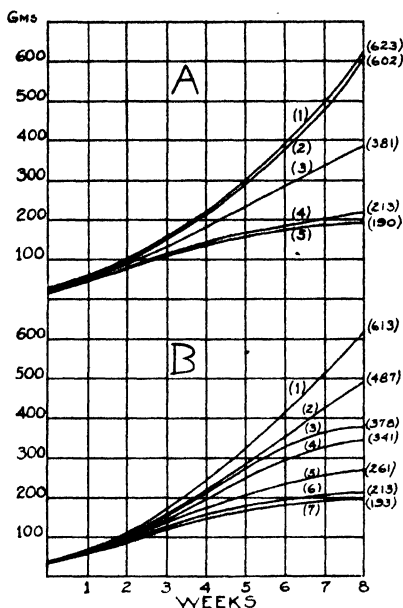


FIG. 1. The figures represent average growth curves for groups of chickens receiving the same basal ration plus the following supplements per 100 gm. of diet. A, Curve 1, 37 i.u. of vitamin D from cod liver oil, 120 i.u. of vitamin A; Curve 2, 37 i.u. of vitamin D from cod liver oil, 652 i.u. of vitamin A; Curve 3, 10 i.u. of vitamin D from cod liver oil, 120 i.u. of vitamin A; Curve 4, 0 unit of vitamin D and 652 i.u. of vitamin A; Curve 5, 0 unit of vitamin D and 120 i.u. of vitamin A. B, Curve 1, 37 i.u. of vitamin D from cod liver oil; Curve 2, 37 i.u. of vitamin D from Tuna Liver Oil 1; Curve 3, 100 i.u. of vitamin D from irradiated ergosterol; Curve 4, 10 i.u. of vitamin D from cod liver oil; Curve 5, 10 i.u. of vitamin D from Tuna Liver Oil 2; Curve 6, 10 i.u. of vitamin D from Tuna Liver Oil 1; Curve 7, 0 unit of vitamin D. The figures in parentheses denote the final average weights of the groups.

In order to compare, in a different manner, these oils with cod liver oil as sources of vitamin D, it was decided to feed similar groups of chickens over a longer period of time and see whether the oils would have any pronounced effect on body weight.

Hart and coworkers (16) early indicated that sunlight had a

growth-stimulating effect on chickens; since that time numerous publications have mentioned the growth-promoting influence of vitamin D. However, few data are available on the quantitative relationships of vitamin D to growth of chicks. For this reason a preliminary experiment was conducted as follows.

Chicks were fed the same basal diet as in the previous experiments. This ration seemed to be adequate in all known vitamins with the possible exception of vitamin A. Three groups of chickens were started on this feed supplemented with 0, 10, and 37 international units respectively of vitamin D per 100 gm. of diet from cod liver oil. These diets were adjusted so that all of the groups received 120 i. u. of vitamin A per 100 gm. of ration in excess of that inherent in the basal mixture. Two additional groups were started on supplement levels of 0 and 37 i. u. of vitamin D, but these diets were adjusted to contain 652 i. u. of vitamin A per 100 gm. above that in the basal ration.

The results of this experiment are shown by the growth curves, A, Fig. 1. It can be seen from these curves that vitamin D, in the presence of all other known vitamins, exerts a definite influence on the body weight of the chick. Since those groups which received the larger quantity of vitamin A showed the same growth responses as the birds on the lower level, it seemed apparent that the basal ration required little or no extra vitamin A for satisfactory growth.

A similar series of investigations next was conducted in which vitamin D from several sources was incorporated in the basal ration. The sources and levels fed are recorded in the legend to Fig. 1; the growth curves in B show the results obtained. The data from this experiment indicate that rat unit for rat unit the several sources of vitamin D tested are considerably less effective in the promotion of growth with the chicken than is cod liver oil.

SUMMARY

1. Several commercial samples of tuna liver oil have been shown to be only 40 to 60 per cent as effective as cod liver oil, rat unit for rat unit, as measured by the bone ash of chickens.

2. These same sources of vitamin D also have been demonstrated to be much less efficient than the vitamin D from cod liver

oil in the promotion of growth in the chick during the first few months of life.

3. It is indicated that vitamin D has a definite influence on the body weight of the chick when supplemented in a diet adequate in all other known vitamins.

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STUDIES ON THE RELATIVE EFFICIENCY OF VITAMIN D FROM SEVERAL SOURCES

II. INFLUENCE OF VITAMIN D OF DIFFERENT ORIGINS ON THE SERUM PHOSPHATASE OF THE CHICKEN

BY JOHN T. CORRELL AND E. C. WISE

(From the Research Laboratories, The Upjohn Company, Kalamazoo)

(Received for publication, August 26, 1938)

In Paper I of this series, Correll and Wise (1) have reviewed some of the literature pertaining to investigations of vitamin D by the dual rat-chick assay technique. They also submit data which indicate that the antirachitic substances from various sources exert different degrees of influence on the growth of the chick as measured by body weight as well as on bone calcification.

Clinical investigations by Bodansky and Jaffe (2), Barnes and Carpenter (3), and others indicate that the phosphatase concentration of the serum is directly related to the presence or absence of vitamin D. In cases of active rickets the phosphatase concentration is markedly increased, while in the presence of an adequate dosage of vitamin D the enzyme showed a normal low value.

In the light of these results it appeared of interest to determine how the several antirachitic materials, which have been demonstrated to exert varying degrees of influence on ossification and growth in the chick, might compare in their influence on the mineral metabolism of the chick as measured by the phosphatase content of the serum.

Very few data have been published concerning phosphatase values in chicken serum. Common (4), using Bodansky's method, reports serum phosphatase concentrations in chickens at different stages of the reproductive cycle. Auchinachie and Emslie (5) have also published results of investigations on the phosphatase activity in the adult fowl. Recently, Peterson and Parrish (6) have reported their findings in studies on the phosphatase con-

centration in the plasma and whole blood of cockerels at 5 and 16 months of age and in hens during the cycle of one egg.

In all of these experiments, however, the birds were fed "normal" diets and no particular consideration was given to the type or quantity of vitamin D consumed. In general, the chickens received more than an adequate amount of vitamin D from cod liver oil; hence the phosphatase values which have been reported undoubtedly represent "normal" values for birds of that age. Inspection of these papers reveals that the average phosphatase values for the mature bird are high when compared to published values for adult humans. Usually the phosphatase is reported as over 20 Bodansky units per 100 cc. of serum or plasma for the adult chicken as compared to 2 to 4 Bodansky units in the blood serum of a healthy mature human. As recorded in the following data, we too found this greater concentration of phosphatase to be the rule in the normal chicken.

It is the purpose of this paper to report our results on a series of investigations comparing the effect of various levels of vitamin D from several sources on the serum phosphatase concentration in the chick.

EXPERIMENTAL

The assay of oils, supplementing of a basal diet, and organization of chicks into suitable groups were all carried out as described in Paper I (1).

The method employed for serum phosphatase determinations was that of Bodansky (7), as was also the calculation and definition of a unit. It was our experience that the sodium β -glycerophosphate substrate would undergo an appreciable hydrolysis on standing even a short time, resulting in considerable color in the reagent blank. For this reason the buffered substrate was made up fresh for each day's determinations.

Blood samples were obtained from the chicks by cardiac puncture. About 3 cc. were taken from four or five individuals in an assay group to make a pooled sample of blood which was considered to be representative of the group. The blood was allowed to clot and serum obtained after centrifugation. Inorganic phosphorus and phosphatase determinations were completed on the day the blood samples were drawn.

To check our technique, analyses were made from time to time on serum from healthy adult subjects. The phosphatase values on these individuals were always found to be around 2.6 units per 100 cc. of serum. These figures are in good agreement with Bodansky's (7) published data of 1.5 to 4.0 units of serum phosphatase for normal adults.

Results

Serum phosphatase concentrations were first determined on four groups of chicks that had been fed varying levels of vitamin D for a period of 21 days. Previous to this the phosphatase had been run on a pooled sample of blood from several of these chicks when they were being started on assay at the age of 2 days. At

TABLE I
Serum Phosphatase Concentrations in Chicks 21 Days of Age Fed Varying Levels of Vitamin D from Several Sources

Group No.	Vitamin D supplement per 100 gm. diet	Phosphatase per 100 cc. serum		
		Male	Female	Average
	<i>international units</i>	<i>units</i>	<i>units</i>	<i>units</i>
143	0	141.8	138.1	140
144	9. Cod liver oil	80.1	110.1	95.1
146	27. " " "	43.5	40.7	42.1
150	9. Tuna Liver Oil 1	130.8	126.5	128.7

this time they showed 81 units of phosphatase per 100 cc. of serum. The blood of a normal 3 month-old cockerel was also investigated at this time and found to have 30 units of phosphatase per 100 cc. of serum.

To determine whether sex would influence the results of the phosphatase experiments these blood samples were divided into pooled samples from males and females for each group.

The data in Table I indicate that varying levels of vitamin D cause a pronounced difference in the concentration of serum phosphatase and, by comparison of Groups 144 and 150, the tuna liver oil appeared to be less effective than the cod liver oil in reducing the serum phosphatase. However, the level of 9 international units used as a basis of comparison was unsatisfactory, as 9 i. u. of vitamin D per 100 gm. of ration, even from cod liver oil, are

known to be inadequate for protection as measured by bone ash. Thus the enzyme concentration increased slightly in Group 144 from the original value of 81 units; it rose considerably higher in Groups 143 and 150 which had received less efficient antirachitic supplements. Group 146 had been given sufficient vitamin D from an efficient source and showed a decrease in serum phosphatase from 81 to 42 units per 100 cc. of serum.

There appeared to be no variation consistently favoring either sex, and from these data it seemed reasonable to use pooled blood samples from future groups, each sex being evenly represented in every sample.

Serum phosphatase values were determined on several groups of chickens 21 days of age that had been fed a ration supplemented

TABLE II

Serum Phosphatase Concentrations in Chicks 21 Days of Age Fed Same Level of Vitamin D from Several Sources

Group No.	Vitamin D supplement, 27 i.u. per 100 gm. diet from	Phosphatase per 100 cc. serum
		units
153	No supplement	231.8
156	Cod liver oil	46.8
159	Tuna Liver Oil 2	81.4
162	" " " 1	71.2

with an adequate amount of vitamin D from cod liver oil as demonstrated by previously reported bone ash data, and on other groups that had been receiving an equal quantity of vitamin D from tuna liver oils. The results of this work are presented in Table II.

Data in Table II indicate that the cod liver oil was more efficient in reducing serum phosphatase concentration of the chick 21 days of age than was the same level of vitamin D from the tuna liver oils employed.

Since serum phosphatase concentration has been shown to decrease as the organism matures under the influence of a "normal" diet, it appeared of interest to determine what effect vitamin D from the above sources would have on chicken phosphatase when the birds were reared on such diets for several months.

Table III includes the data accumulated from the phosphatase analysis of birds reared on several sources of the antirachitic vitamin over a period of 13 weeks. In Groups 8 and 12, at the 8th week the tuna liver oil again appears less efficient than cod liver

TABLE III

Serum Phosphatase Concentration in Chicks 8 to 13 Weeks of Age Fed Vitamin D from Several Sources

	Group 6	Group 8*	Group 12*		Group 15*		
	Vitamin D per 100 gm. diet, 1st to 8th wk.						
0	37 i.u., cod liver oil	37 i.u., Tuna Liver Oil 1	100 i.u., irradiated ergosterol				
	Phosphatase per 100 cc. serum at 8th wk.						
units	units	units		units			
220.4	45.3	100.7		187.1			
	A	B	A	B	A	B	
	Vitamin D per 100 gm. diet, 9th to 13th wk.						
0	37 i.u., cod liver oil	37 i.u., Tuna Liver Oil 1	37 i.u., Tuna Liver Oil 1	37 i.u., cod liver oil	100 i.u., irradiated ergosterol	37 i.u., cod liver oil	
	Phosphatase per 100 cc. of serum						
units	units	units	units	units	units	units	
9 wks.	196.9	33.0	34.4	84.7	51.7	175.9	104.2
10 "	188.7	29.3	43.7	72.7	37.7	173.0	45.3
11 "	178.9	41.0	59.1	119.4	36.8	190.4	71.3
12 "	157.0	29.0	66.5	90.0	29.7	185.3	47.9
13 "	Sample lost	22.9	64.5	93.3	25.7	193.1	26.1
Approximate change since 8th wk.	No real change	-22.4	+19.2	No real change	-75	No real change	-161

* Groups 8, 12, and 15 were divided into two groups each, 5 birds per group, at the 8th week.

oil in its influence on calcium and phosphorus metabolism as measured by the phosphatase concentration of the serum. Irradiated ergosterol (Group 15) was even less effective. To check this further, several of the groups of chickens were divided. Half of those supplemented with cod liver oil were continued on this

supplement; the other half were changed over to a tuna liver oil adjuvant. Likewise, the tuna liver oil and irradiated ergosterol groups were divided so that half of them continued on the original diet, the other half receiving an adequate dosage of vitamin D from cod liver oil.

As will be seen by referring to Table III, those birds which were fed cod liver oil showed declining serum phosphatase concentrations to normal ranges, even in the groups that had had high values from receiving an inefficient diet for the first 8 weeks (Groups 8A, 12B, and 15B). Chicks changed at the 8th week from cod liver oil to an equal dosage of vitamin D from tuna liver

TABLE IV

Influence of Vitamin D from Several Sources on Serum Phosphatase Concentrations in Growing Chick

	Group 23	Group 25	Group 26	Group 28	Group 29	Group 34
	Vitamin D per 100 gm. diet					
	0	18 i. u., cod liver oil	37 i. u., cod liver oil	18 i. u., Tuna Liver Oil 1	37 i. u., Tuna Liver Oil 1	60 i. u., Tuna Liver Oil 1
	Phosphatase per 100 cc. serum					
	units	units	units	units	units	units
1st day	71.3	71.3	71.3	71.3	71.3	71.3
2 wks.	158.7	56.4	69.6	93.0	81.3	60.0
4 "	267.7	44.1	41.4	103.4	65.0	45.1
6 "	248.0	54.8	48.2	156.8	115.2	46.8
8 "	240.0	44.0	35.6	157.9	76.6	29.4

oil demonstrated a marked increase of serum phosphatase over the low value obtained from the previous cod liver oil supplement (Group 8B). When the birds were reared the entire 13 weeks on tuna liver oil or irradiated ergosterol supplements, high serum phosphatase concentrations resulted (Groups 12A and 15A).

In our most recent investigation we have attempted to determine how many units of vitamin D from tuna liver oil are required by the chick to give phosphatase responses equivalent to those obtained from an adequate dosage of vitamin D from cod liver oil. The results of this work are recorded in Table IV.

The data in Table IV seem to indicate that at about 18 i. u. of vitamin D from cod liver oil the minimum effective level as

measured by phosphatase concentration is reached. It has been our experience that this is the level of cod liver oil which also yields a "normal" bone ash. When tuna liver oil was used at a level of 60 i. u. per 100 gm. of ration, the phosphatase values obtained indicated such a level to be adequate. A level of around 50 i. u. from this source might prove sufficient.

The data accumulated in these investigations indicate that the tuna liver oils employed were approximately 30 to 40 per cent as effective in reducing the phosphatase concentration as was the vitamin D from cod liver oil; irradiated ergosterol was even less efficient at the levels administered. These results are in agreement with conclusions derived from comparative bone ash determinations and add additional support to the prevailing theory that the antirachitic vitamin exists in several different forms. There remains the possibility that such materials as cod liver oil may contain some factor in addition to the vitamin D entity which adds to its antirachitic effectiveness. In any case serum phosphatase determinations on the chick offer another reasonably sensitive and convenient method for measuring differences in physiological activity between the increasing number of substances said to possess antirachitic activity.

SUMMARY

1. The phosphatase concentration of the 2 day-old chick has been shown to be around 80 Bodansky units per 100 cc. of serum.

2. Under the influence of an adequate amount of vitamin D the high serum phosphatase concentrations of the growing chick decrease, approaching a normal level of 20 units per 100 cc. of serum for adult birds.

3. In the absence of vitamin D in the diet the phosphatase concentration has been shown to rise to values of over 200 units for highly rachitic chicks.

4. The antirachitic vitamin from several sources has been demonstrated to exert varying degrees of influence on the serum phosphatase values in chicken serum. Cod liver oil was more efficient than irradiated ergosterol and the tuna liver oils employed in reducing the phosphatase activity in the serum of the growing chick.

5. It is suggested that serum phosphatase determinations on

chicks after the administration of various forms of vitamin D offer another method for measuring differences and similarities among the numerous antirachitic substances.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF INULIN IN BLOOD PLASMA AND URINE

By KURT STEINITZ

(From the Department of Internal Medicine of Istanbul University, Guraba Hospital, Istanbul, Turkey)

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Of all substances used in the determination of glomerular filtration, inulin is one of the most important. Hendrix, Westfall, and Richards (1) consider it to be the most suitable for the calculation of the filtration rate, because it is the substance with the greatest molecular weight known so far which is completely filtered through glomerular membrane (Westfall and Landis (2), Bunim, Smith, and Smith (3)). Inulin combines the ideal properties required in a substance used for the calculation of glomerular filtration (Jolliffe, Shannon, and Smith (4)). It is probable that inulin, an inert substance which mixes with the blood and body fluid without penetrating into the cells (Van Slyke, Hiller, and Miller (5)), will also become important in certain other physiological studies. For these reasons it is desirable to have a sensitive and exact quantitative test for the estimation of inulin.

So far inulin has been estimated by the difference of reduction before and after hydrolysis of blood plasma filtrate. Fasting blood, however, gives also a difference of reduction before and after hydrolysis. This fasting value must be subtracted from the results obtained. According to Van Slyke, Hiller, and Miller (6) this difference in dogs is always under 5 mg. per cent, calculated as glucose. In man, however, this difference in blood filtrate can attain considerably higher values. With the technique of Van Slyke and his collaborators (6),¹ values between 3 and 25 mg. per

¹ The following remark should be made on the technique of these authors, who propose to accomplish precipitation with $\text{Cd}(\text{OH})_2$. In experiments in which inulin was added to plasma the precipitation was made either with $\text{Zn}(\text{OH})_2$ or with $\text{Cd}(\text{OH})_2$. In four cases the filtrates of the $\text{Zn}(\text{OH})_2$ pre-

cent were found, which agree with the values reported by Pavy (9), Frank and Bretschneider (10), Stasiak (11), and Everett and Sheppard (12). Such high blank values can form a disproportionately great part of the inulin value. This disproportion makes the estimation of small quantities of inulin with reduction methods rather uncertain. Therefore only values above 50 mg. per cent are described and evaluated.

For experiments in man, however, the use of larger quantities of inulin is very troublesome because of its intravenous administration. For these reasons another principle, still allowing the estimation of 5 mg. per cent of inulin, has been tried. This procedure depends on the colorimetric determination of levulose formed from inulin during acid hydrolysis according to Roe's quantitative method, based on the Seliwanoff reaction (13). The 10 *N* hydrochloric acid used in the reaction hydrolyzes the inulin so rapidly that the full levulose color develops during the short reaction time, and so the period of hydrolysis is reduced from 2 hours to a few minutes. A green filter is used for the colorimetric or photometric comparison.

Reagents and Procedure

All reagents are made according to Roe's prescriptions (13). For plasma determinations, however, the following dilutions of the standard solution of levulose are required: 0.5, 1, 2, and 3 mg. per cent in saturated benzoic acid. For the estimation of urine dilutions of 5, 10, 20, and 30 mg. per cent in 1 per cent acetic acid with 1 per cent sublimate are prepared. These dilutions remain stable for some weeks.

Both for plasma and urine the technique of Roe is used. Before the inulin is injected the blank values of the fasting plasma and urine are determined. 2 cc. of the standard dilutions are treated in the same manner. Read the unknown against the standard which most closely matches it. A green filter of 510 *mμ* is used.

cipitation gave evidently higher inulin values. Similar objections against the $\text{Cd}(\text{OH})_2$ precipitation of Fujita and Iwatake (7) are also made by Rappaport (8).

DISCUSSION

The conditions of Roe's method have been reexamined.

1. The maximum of the absorption is to be found at 510 m μ (examined with a Leitz photometer).

2. The Beer-Lambert law is applicable only in narrow ranges of concentration. If the concentration rises, the depth of color increases less than the concentration.

Levulose mg. per cent	Extinction coefficient
10	0.92
20	1.64
50	3.45
100	5.38

Because of these obvious deviations from the Beer-Lambert law it is necessary to use several of the standard dilutions, *e.g.* 0.5, 1, 2, and 3 mg. per cent, for plasma determinations, and to compare the unknown with the dilution that most closely matches it.

3. The depth of the color depends, as Roe has shown, on the quantity of alcohol, on the concentration of the hydrochloric acid, and on the temperature of the water bath and time the tubes remain in the bath. As it is impossible to maintain these conditions with sufficient accuracy, one cannot make a comparison curve, because exactly the same color is not obtainable in repeated tests. It is necessary to treat all the standards at the same time and in the same manner as the unknown.

4. In the first tests heating was continued for 2 hours with 1 cc. of *N* hydrochloric acid at 80°. Later in order to simplify the procedure, we tried to find out whether the 10 *N* hydrochloric acid completed the hydrolysis during the 8 minutes reaction time. To each test-tube were added 2 cc. of inulin-containing plasma filtrate and 1 cc. of *N* HCl; the tubes were placed in the water bath and heated for 10, 20, 30, up to 120 minutes. All the tubes gave exactly the same color. High inulin concentrations with and without previous heating also gave the same result, which indicates that the 10 *N* hydrochloric acid at 80° hydrolyzes inulin, even in high concentrations, so quickly that the full development of levulose color is assured after a reaction time of 8 minutes.

5. Inulin gives the same depth of color in the same concentrations as levulose.

<i>mg. per cent</i>	<i>Inulin extinction coefficient</i>	<i>Levulose extinction coefficient</i>
10	0.92	0.92
50	3.45	3.45
100	5.38	5.38

Duplicate determinations nearly always give identical results, even in low concentrations, if the same conditions are maintained.

6. It had to be proved whether in urine analyses the treatment with charcoal in the acetic acid medium removes a part of the inulin. For this purpose two series of tests were made. In one of them the sample was first treated with charcoal and then hydrolyzed with 1 cc. of N HCl; in the second the hydrolysis was made first, followed by the charcoal treatment. Both series led to identical results, showing that primary carbon treatment does not precipitate any inulin.

7. The fasting value in the plasma with normal blood sugar level corresponds to 1 to 2 mg. per cent of levulose; in urine the fasting value varies from 10 to 20 mg. per cent.

These facts confirm the accuracy of Roe's method for the determination of inulin in plasma and urine. Experiments in which inulin was added to plasma or urine gave an error of ± 4 per cent compared with the levulose standard. It must be remarked, however, that results in duplicate determinations are always identical, so that we may suppose that the error has not been caused by the color reaction, but by some other factors; *e.g.*, by the water content of the inulin sample or by inaccuracy of the dilution, etc. With this colorimetric method determinations of inulin may easily be made even in low concentrations. For the determination of the glomerular filtration it is sufficient to give a man of 70 kilos 10 gm. of inulin in order to obtain plasma values of 20 to 40 mg. per cent after 1 hour.

SUMMARY

The present methods for the determination of inulin in plasma and urine are criticized.

A simple colorimetric method based on Roe's levulose determination is proposed to measure the levulose formed by hydrolysis of inulin. Low inulin concentrations may be exactly determined.

It is shown that the time of hydrolysis may be shortened considerably, as the inulin is rapidly hydrolyzed.

The conditions of the color reaction are discussed.

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FURTHER STUDIES ON THE ESTIMATION OF SMALL AMOUNTS OF SODIUM PREGNANEDIOL GLUCURONIDATE IN URINE

By ELEANOR HILL VENNING

(From the Department of Medicine, McGill University Clinic, Royal Victoria Hospital, Montreal, Canada)

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In 1937 (1) a method for the gravimetric determination of sodium pregnanediol glucuronide was described by the author. Since then over 2000 determinations have been carried out on urine of pregnancy and urine of the menstrual cycle and considerable experience has been acquired during this time. On account of the wide interest shown in the method and its application to various problems, particularly to disorders of the menstrual cycle, it has been thought advisable to report further improvements in the technique and also to discuss in detail the difficulties encountered.

Although pure crystalline precipitates of sodium pregnanediol glucuronide can be obtained consistently from pregnancy urine, this is not always the case when small amounts are extracted from urines of the menstrual cycle and certain reservations must be made when applying this method to the latter. The reason for this is that when large volumes of urine have to be extracted in order to obtain a measurable amount of sodium pregnanediol glucuronide greater amounts of impurities are carried through to the end and are not diluted out as in the case of pregnancy urine. These impurities are not proportional to the amount of urine extracted but vary with different specimens. In about 60 per cent of the analyses they are negligible, 0.2 to 0.5 mg.; in the remainder, however, they may vary anywhere from 1 to 10 mg. As this residue often appears to be similar to the sodium pregnanediol glucuronide precipitate, care must be taken not to mistake it for this compound. For this reason it is felt that in the case of

urines that contain less than 5 mg. of sodium pregnanediol glucuronidate per liter this method should be used as a qualitative test rather than as an accurate gravimetric method.

Collection of Urine and Use of Preservatives—Certain precautions must be taken in the collection of the urine itself. Complete 24 hour specimens are collected in large bottles containing a preservative. If the extraction with butyl alcohol is not being carried out immediately, the urine should be kept in a refrigerator, as it has been found that hydrolysis of the sodium pregnanediol glucuronidate to free pregnanediol occurs in urine at room temperature even in the presence of a preservative, thus causing a loss in the final value. The efficiency of preservatives such as tricresol, toluene, chloroform, and NaCN in preventing hydrolysis has been investigated. If the urines are kept chilled at 4–7°, no hydrolysis occurs even up to a period of 11 days. However, at higher temperatures (18–34°) a splitting of the compound occurs even in the presence of these preservatives. Tricresol (6 drops per 24 hour volume) appears to have a better preserving power than either toluene (3 cc.) or chloroform (3 cc.). 0.5 per cent of NaCN will prevent the enzymic hydrolysis of the compound at 20° for a period of at least 4 days; however, on further standing hydrolysis does occur to some extent. At temperatures ranging between 26–34°, 0.5 per cent of NaCN fails to prevent hydrolysis. Lower concentrations of cyanide in the urine give proportionately lower recoveries for the same temperature.

Amount of Urine Extracted—The amount of urine taken for the analysis plays an important rôle in the purity of the final precipitate and it has been found that in pregnancy urine the recoveries are most satisfactory when the volume analyzed contains between 10 and 15 mg. of sodium pregnanediol glucuronidate (*i.e.*, 6 to 9 mg. calculated as pregnanediol). The excretion of pregnanediol during pregnancy has been reported by Browne, Henry, and Venning (2). In Fig. 1 are charted the variations occurring in the daily excretion of pregnanediol throughout pregnancy (values taken from eight cases of normal pregnancy). The necessary amount of pregnancy urine to be extracted can be approximated from this chart. It is dependent upon the total 24 hour volume as well as the duration of pregnancy. In urine of the menstrual cycle it is necessary to extract the full 24 hour specimen and in

those cases in which only a few mg. of the compound are being excreted, a 48 hour specimen should be used. In order to obtain good recoveries the sample of urine should contain at least 4 to 5 mg. of sodium pregnanediol glucuronide. The amounts of pregnanediol excreted in the menstrual cycle have been already reported in detail by Venning and Browne (3) and by Wilson, Randall, and Osterberg (4).

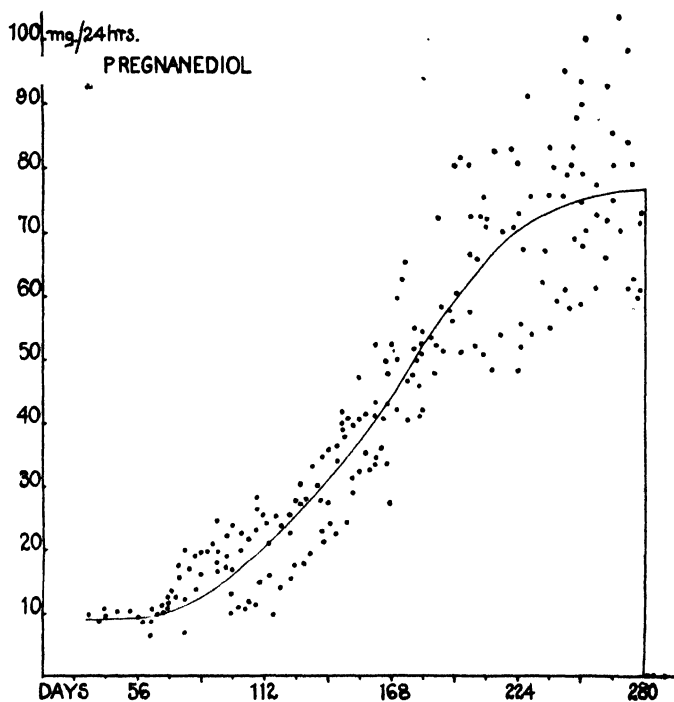


FIG. 1. Urinary excretion of pregnanediol in normal pregnancy (eight cases).

The urine should not be acidified, as this tends to facilitate the extraction of a gummy material which is very difficult to remove and interferes with the subsequent precipitation of the sodium pregnanediol glucuronide.

Extraction of Urine with Butyl Alcohol—Care must be taken to prevent the formation of an emulsion when the urine is extracted

with butyl alcohol. The mixture should not be shaken vigorously but only gently mixed with a rotary motion, each extraction requiring approximately 1 minute of shaking. Four extractions in all are made. In spite of precautions some urines do form an emulsion, particularly those obtained from cases of toxemia of pregnancy, and in order to separate the butyl alcohol, the mixture must be centrifuged. The formation of an emulsion tends to cause a loss of the pregnanediol compound, unless the urine separating out from the centrifuging of the emulsion is extracted again with butyl alcohol.

The butyl alcohol should be clear before it is evaporated to dryness. If there is a residue at the bottom, the clear butyl alcohol is poured off and the residue is extracted with fresh butyl alcohol. This is added to the first fraction. The combined butyl alcohol is then taken to dryness under reduced pressure. When a vacuum pump is used and the evaporation flask is kept in a boiling water bath the butyl alcohol distils over rapidly. The temperature of the boiling butyl alcohol under these conditions remains between 40–49°. The residue in the flask is then dissolved in 60 cc. of 0.1 N NaOH.

Second Extraction with Butyl Alcohol—This alkaline solution is extracted four times with butyl alcohol. The combined fractions of butyl alcohol are allowed to stand until clear, as some residue usually settles out. The clear butyl alcohol is poured back into the separatory funnel and the residue is washed twice with small amounts of butyl alcohol which are added to the funnel. If the combined butyl alcohol is washed once with 3 cc. of 0.1 N NaOH, before the two washings with water (5 cc.), an increased amount of coloring matter is removed. After each washing the mixture is allowed to stand for complete separation of the two layers. The butyl alcohol is again evaporated to dryness under reduced pressure.

Precipitation with Acetone—The first precipitation is carried out as follows: Exactly 5 cc. of water are added to the flask with about 10 cc. of acetone. The contents are dissolved in this mixture and are transferred to a 125 cc. Erlenmeyer flask. The final volume of the mixture is made up to 100 cc. with acetone and it is allowed to stand overnight. A precipitate settles out. Most of the supernatant fluid can be drawn off by suction and the re-

mainder is centrifuged. After centrifugation the acetone is poured off without disturbing the precipitate, leaving the residue in the tube.

A second precipitation with acetone must always be made. At this stage a modification in the procedure has been introduced which helps further to eliminate impurities which have been carried through to this point. The amount of water to be added for the second precipitation is dependent upon the weight of the precipitate. This can easily be approximated. If it is 10 mg. or more, use 5 cc. of water, if between 5 and 10 mg., use 3 cc. of water, and if less than 5 mg., use 2 cc. of water. The required amount of water is measured into the Erlenmeyer flask and an equal volume of acetone is added. The mixture is warmed to dissolve the precipitate which adheres to the surface of the flask. The solution is then transferred to the centrifuge tube and the remaining precipitate is dissolved by heating. This mixture is cooled and is filtered back into the Erlenmeyer flask with suction. On cooling, a considerable amount of impurities comes out of solution but the small amount of Na pregnanediol glucuronidate present remains in solution. Filtering the mixture at this stage is of particular value in the case of urines from the menstrual cycle. However, when the first precipitate is relatively pure, as in the case of pregnancy urine, it may be omitted. Acetone is then added to the filtrate to bring the volume up to 100 cc. and it is allowed to stand overnight in the refrigerator. After standing, the supernatant fluid is again drawn off and the remainder is centrifuged. If the precipitate does not settle down compactly but remains in suspension, the remaining fluid should be filtered. The precipitate is dissolved in hot ethyl alcohol. Occasionally a small amount of water has to be added to insure complete solution. The hot alcohol is filtered into a weighed beaker, evaporated to dryness on a water bath, and the contents weighed. A melting point is taken.

Identification of Precipitate—The final precipitates obtained from pregnancy urines are usually quite pure and crystalline, but those derived from urines of the menstrual cycle cannot be accepted as representing pure sodium pregnanediol glucuronidate, owing to the fact that large volumes of urine have to be extracted in order to obtain measurable amounts of the compound. These

precipitates always contain varying amounts of impurities and for this reason a melting point must always be taken. If the melting point is between 271–260°, the amount of impurities probably varies between 0 and 10 per cent; if below 260°, the impurities are accordingly increased. The melting point of the precipitate must be taken into account before the results can be interpreted.

Usually if the precipitate does not melt or decompose when heated to 280°, it has been regarded as not containing any sodium pregnanediol glucuronidate. However, recently a precipitate weighing 7.4 mg. was obtained from 2 liters of urine which failed to melt at 280°. The precipitates from 6 days excretion were combined (total 23.4 mg.) and were purified by recrystallization from acetone and water and alcohol. A crystalline material weighing 7 mg. was obtained which melted at 268° with evolution of gas. The impurities in this mixture were equal to about 70 per cent and were great enough to obscure the presence of the compound, unless repurification had been carried out.

With the present technique the weight of the final residue in negative urines (1000 to 1500 cc.) in the majority of cases varies from 0.2 to 1 mg. However, occasionally certain negative urines do give residues greater than this. In one extreme case residues weighing 44 mg. and 20 mg. were obtained in two 48 hour periods. The melting points were 80° and 110° respectively. No evolution of gas was noticed. When the residues were dissolved in 0.1 N NaOH, reextracted with butyl alcohol, and precipitated with acetone, no precipitate was obtained.

Recovery of Small Amounts of Sodium Pregnanediol Glucuronidate—The recovery of 1, 2, 3, and 5 mg. of sodium pregnanediol glucuronidate in 1 liter of urine was measured, 2 cc. of water being used for the second precipitation. (The results are shown in Table I.) It was impossible to detect a total amount of 1 mg. of sodium pregnanediol glucuronidate at any time. The recovery of 2 mg. was variable. In some urines this amount could not be detected, in others 40 to 60 per cent was recovered. 3 mg. could always be detected, the recovery ranging from 50 to 67 per cent; when 5 mg. were added, the recovery was 70 to 75 per cent. Therefore in order to detect small amounts of the compound in urine from the menstrual cycle, particularly in abnormal cases in which the amounts are usually low, sufficient urine must be ex-

tracted so that the total content of the sodium pregnanediol glucuronidate in the sample is at least 4 to 5 mg. It may be necessary to unite several days specimens in order to obtain this amount. As the recovery of these small amounts varies somewhat with different urines, the results can only be approximated.

TABLE I
Recovery of Small Amounts of Sodium Pregnanediol Glucuronidate from Urine

Sodium pregnanediol glucuronidate added	Weight of ppt.	Melting point	Impurities (calculated)	Recovery
mg.	mg.	°C.	per cent	per cent
0	0.8	110	100	
1	0.8	110	100	0
2	2.0	220	40	60
3	2.8	245	29	67
5	4.5	252	18	74

TABLE II
Recovery of Sodium Pregnanediol Glucuronidate from Urine

Weight of ppt.	Recovery		
	2 cc. water (approximate)	3 cc. water	5 cc. water
mg.	per cent	per cent	per cent
2	0-60		
3-4	50-67		
5-8	70-75	79	
9-10		82	75
11-12		85	78
13-15			81
16-18			83
19-25			85

Calculation—The variation in the percentage recovery depending upon the amount of compound originally present and the volume of water used in the second precipitate is shown in Table II. The calculated amount of sodium pregnanediol glucuronidate is multiplied by the factor 0.597 to convert it into terms of pregnanediol, the results being expressed in mg. of pregnanediol excreted per 24 hours.

SUMMARY

The difficulties encountered in the estimation of small amounts of sodium pregnanediol glucuronidate in urine are discussed and improvements in the technique are described.

The author wishes to express her appreciation for the technical assistance rendered by Mr. V. Kazmin.

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A MECHANISM OF SECRETION IN THE CHORIOID PLEXUS. THE CONVERSION OF OXIDATION-REDUCTION ENERGY INTO WORK*

BY ROBERT D. STIEHLER AND LOUIS B. FLEXNER

(From the Departments of Ophthalmology and Anatomy, the Johns Hopkins University, Baltimore)

(Received for publication, May 25, 1938)

In ultrafiltration the maximum concentration change which can be produced between the mother liquor and the ultrafiltrate can be calculated from the pressure of ultrafiltration and the composition of the mother liquor (4). A comparison of the concentrations of diffusible substances in blood plasma and cerebrospinal fluid indicates that capillary blood pressure does not suffice to account for the concentration differences between these two fluids and that secretory work, therefore, is necessary for the formation of cerebrospinal fluid (3). Previous investigations (2) show that the chorioid plexus is the predominant source of the fluid. Consequently the chorioid plexus must supply the secretory energy necessary for the formation of cerebrospinal fluid.

The studies reported here have been directed towards elucidating the source of this energy in the plexus and the mechanism for transforming this energy into secretory work. Similar studies have been previously reported (7) for the secretory mechanism of the ciliary body of the eye. In the ciliary body, investigations were made of the oxidation-reduction potentials of epithelium and stroma, of the distribution of indophenol oxidase, and of the barrier between epithelium and stroma. These observations led to the postulation of an electric current between stroma and epithelium. Similar experiments and conclusions are reported here for the chorioid plexus.

* This investigation was supported in part by a grant from the John and Mary R. Markle Foundation.

Material and Methods

Fetal pigs from the last third of the gestation period, obtained as previously described (6), were used as a source of material. As has been demonstrated (5), distribution ratios in these fetuses have the same characteristics as the adult. Plexuses kept in Ringer's solution (see Section 7) remain viable for from 2 to 3 hours. Where buffers were used, they were isotonic and contained KCl, CaCl₂, MgCl₂, and KH₂PO₄ in the same concentrations as in Ringer's solution.

In this report methods of study will be only briefly indicated under the appropriate headings. These methods have been described in detail by Friedenwald and Stiehler (7). The technique referred to below as "supravital" is as follows: The intact chorioid plexus was removed from the ventricle and laid flat upon a glass slide in the bathing fluid. Such a preparation presented a free, thin edge easily observed microscopically. This free edge was then stained to an appropriate intensity with minute quantities of a chosen dye solution (7).

EXPERIMENTAL

The experimental observations deal with three anatomical elements of the chorioid plexus, namely epithelium, stroma, and the barrier between epithelium and stroma (Fig. 1). Since this barrier cannot be seen microscopically, except as a refractile line, we have not called it a basement membrane but refer to it as the stromal-epithelial barrier.

The different types of experiments performed are recorded separately below. They deal with the distribution of indophenol oxidase between the epithelium and stroma of the plexus, the difference of potential between these two tissues, the passage of dyes across the stromal-epithelial barrier, and the physicochemical characteristics of this barrier.

1. *Distribution of Indophenol Oxidase in Chorioid Plexus*—The presence of indophenol oxidase was tested for with dimethyl *p*-phenylenediamine and α -naphthol (7). After treatment with these reagents the epithelium of the plexus was intensely stained with indophenol blue; the stroma contained no indophenol blue.

2. *Oxidation-Reduction Potentials of Chorioid Plexus*—The

potentials of epithelium and stroma were determined by introducing oxidation-reduction indicators intravascularly or supravitaly. In all instances care was taken to use a minimum of the indicator. Solutions, 0.01 M, were used intravascularly; 0.001 M solutions, supravitaly. With all dyes except the safranines, the potentials were approached from both the reduced and oxidized states of the indicator and were equivalent. The degree of reduction was estimated by the increase in color produced by addition of ferri-cyanide. This was necessary to rule out irreversible changes of the indicator in the tissue (1). With appropriate indicators the

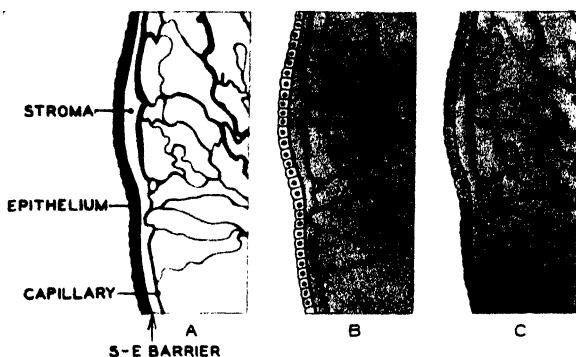


FIG. 1. Illustration of behavior of dyes introduced into chorioid plexus. The shaded areas indicate the presence of dye. *A* represents basic dyes in air; *B*, acid dyes in air; *C*, neutral dyes in air, or acid, basic, or neutral dyes when plexus is asphyxiated. *S-E barrier* represents the stromal-epithelial barrier.

error in the measured potential is thought to be within ± 0.015 volt.

The results of these measurements are shown in Table I. As can be seen from Table I the potential of the epithelium in air at pH 7.4 was +0.100 volt, while the potential of the stroma was -0.130 volt, giving a difference of 0.230 volt. Under N_2 both epithelium and stroma had the same potential, -0.290 volt. In the presence of 0.001 M cyanide and air epithelium and stroma each had a potential of -0.200 volt.

*3. Penetration of Dyes Through Stromal-Epithelial Barrier—*To study the passage of dyes from stroma to epithelium, dye was

introduced into the stroma by intravascular injection. To study the passage of dyes from epithelium to stroma, the supravital technique was used. In those experiments dealing with asphyxia, the tissue was placed either in 0.001 M cyanide solution or in an atmosphere of N_2 . In the case of the investigation of the passage of basic dyes from stroma to epithelium under cyanide anoxia, the lateral ventricles were irrigated with the cyanide solution just

TABLE I
Oxidation-Reduction Potentials of Chorioid Plexus

0 represents no reduction, + one-third reduced, ++ one-half to two-thirds reduced, +++ three-fourths to nine-tenths reduced, ++++ over nine-tenths reduced.

Indicator	E'_{out} pH 7.4	Reduction of indicator under			
		Aerobiosis		Anaerobiosis	
		Stroma	Epithelium	Stroma	Epithelium
	<i>volt</i>				
Toluylene blue	+0.101	++++	++	++++	++++
Lauth's violet	+0.050	++++	0	++++	++++
Methylene blue	-0.002	++++	0	++++	++++
Indigotrisulfonate	-0.099	+++	0	++++	++++
Indigodisulfonate	-0.143	+	0	++++	++++
Cresyl violet	-0.175*	0	0	++++	++++
Dimethyl phenosafranine	-0.275	0	0	+++	+++
Safranine T	-0.298	0	0	+	+
Cresyl violet	-0.175*	+++	+++	(With cyanide anoxia)	

* This potential is in doubt since the cresyl violet sold by the National Aniline and Chemical Company, Inc., is probably different chemically from the cresyl violet measured by Rapkine, Struyk, and Wurmser (9).

prior to and during the period of injection. Care was taken to assure no staining of the epithelium by leakage of dye from the wound into the ventricle.

As is to be noted in Table II basic dyes (dyes which are cations in solution) in air did not pass from epithelium to stroma. When these dyes were introduced into the stroma in low concentrations, they accumulated practically completely in the epithelium. Both of these procedures resulted in a picture shown diagrammatically

in Fig. 1, A. Acid dyes (dyes which are anions in solution), in air, passed rapidly from epithelium to stroma and with time the epithelium cleared (Table II). When introduced into the stroma, only a trace of the acid dye could be seen in the epithelium. These two last procedures gave a picture shown diagrammatically in Fig. 1, B. The only neutral dye (dyes which are uncharged or a zwitter ion in solution), rhodamine B, which was used passed rapidly from stroma to epithelium or *vice versa* and was found in equal concentrations throughout the plexus. The typical ap-

TABLE II

Penetration of Dyes Through the Stromal-Epithelial Barrier of Chorioid Plexus at Physiologic pH

0 represents no penetration, + slow diffusion, ++ rapid diffusion, ++++ accumulation beyond diffusion.

Dye	Chemical type	Ionic type	Penetration normally		Penetration with asphyxia	
			Epithelium to stroma	Stroma to epithelium	Epithelium to stroma	Stroma to epithelium
Crystal violet . . .	Triphenylmethane	Basic	0	++++	+	+
Malachite green . . .	"	"	0	++++	++	++
Cresyl violet	Oxazine	"	0	++++	+	+
Eosin	Fluoran	Acid	++	Trace	++	++
Phloxin	"	"	++	"	++	++
Rose bengal	"	"	++	"	++	++
Brom-phenol blue . .	Sulfonephthalein	"	++	"	++	++
Rhodamine B	Fluoran	Neutral	++	++	++	++

pearance of the plexus with this dye is shown diagrammatically in Fig. 1, C. The dyes listed in Table II were used because they met the following requirements. They were not reduced by the plexus in air; they were not chemically altered by the plexus; and they were capable of passing through the stromal-epithelial barrier under appropriate conditions.

With cyanide or N₂ asphyxia, acid, basic, and neutral dyes gave pictures essentially like rhodamine B in air (Table II and Fig. 1, C). This neutral dye behaved under asphyxia as it did in air.

Acid dyes passed rapidly from epithelium to stroma both in air and under asphyxia. The significant experiments with acid dyes under asphyxia involved their passage from stroma to epithelium. In these experiments the stroma was filled with dye by intravascular injection; the plexus was then removed and placed under the microscope for observation. When asphyxiated by cyanide or N_2 , a flash of color was seen in the epithelium. Dye did not accumulate in the epithelium but was leached by the bathing fluid. The concentration of dye in the stroma progressively decreased until the color of stroma and epithelium became of equal intensity. In the case of basic dyes if the stroma were filled while the plexus was irrigated with cyanide solution, the dye slowly diffused into the epithelium until the color intensity of stroma and epithelium was alike. In these instances the dye passed much more slowly than in air and did not accumulate in the epithelium. In another series of experiments, the epithelium was filled in air with basic dyes by the supravital technique. When these preparations were asphyxiated by cyanide or N_2 , dye passed into the stroma and reached a concentration equal to that of the epithelium within 5 minutes. One of the most vivid experiments to show the difference in behavior of basic dyes in air and under asphyxia was performed with malachite green and N_2 . In air this dye accumulated in the epithelium. Under N_2 the dye was quickly distributed in equal concentration between stroma and epithelium. On readmission of air the dye again accumulated in the epithelium. This phenomenon could be repeated.

In all the above experiments the barrier to passage of dye was at the interface between epithelium and stroma. This may be taken as evidence that the stromal-epithelial barrier is different from the membrane at the free epithelial edge. This is also substantiated by the experiments on the effect of pH (Section 5). The experiments on oxidation and reduction (Section 6) demonstrated that this stromal-epithelial barrier is a material entity.

4. *Effect of pH on Indophenol Oxidase and O_2 Consumption of Choroid Plexus*—The indophenol oxidase reaction was studied in plexuses bathed with various pyridine-pyridine acetate buffers. Since the reagents for the reactions are unaffected in these buffers, the activity of the oxidase could be judged by the intensity of indophenol blue present in the epithelium after 1 hour. As is shown

in Table III, the activity of the enzyme was slightly reduced at pH 6.9, was feeble at pH 6.2, and was practically zero at pH 5.7.

O₂ consumption was measured in the Warburg apparatus. The O₂ uptake of plexus was measured in Ringer's solution and then isotonic acetic acid-acetate buffer added to change the pH. The pH of the fluid was determined at the end of an experiment with the glass electrode. As is to be seen in Table III, the change in

TABLE III

Variation of Indophenol Oxidase Activity and O₂ Consumption with pH
++++ represents intense activity.

pH.....	5.70	6.17	6.55	6.90	8.32	Ringer's solution
Oxidase reaction	Trace	+	++	+++	++++	++++
O ₂ consumption, c.mm. per mg. dry weight per hr.....		1-2				13

TABLE IV

Determination of Isoelectric Point of Stromal-Epithelial Barrier. Effect of pH on Penetration of Dyestuffs

0 represents no penetration, + penetration.

pH	Epithelium to stroma		Stroma to epithelium	
	Acid dyes	Basic dyes	Acid dyes	Basic dyes
5.5	Trace	+	Trace	+
5.72	"	+		
5.73	+	+		
5.76	+	+		
5.77	+	0		
5.8	+	0	+	0

O₂ consumption with pH paralleled the change in indophenol oxidase activity.

5. *Effect of pH on Stromal-Epithelial Barrier; Determination of Its Isoelectric Point*—These experiments were performed in largest part with the supravital technique with various acetic acid-acetate buffers. A smaller series of experiments involved intra-vascular injections of the dye while the ventricle was irrigated with the buffer. The pH values recorded in Table IV are those of the

bathing fluid as measured by the glass electrode. The justification for assuming that the plexus has the same pH as the bathing fluid has been given elsewhere (7).

As is noted in Section 3 there was little or no difference in passage of acid and basic dyes through the stromal-epithelial barrier at physiological pH under conditions of asphyxia. With decrease in pH, however, the barrier restricted the passage of acid dyes little or not at all, whereas it restricted the passage of basic dyes completely or almost so. This selective permeability, as shown in Table IV, was observed to pH 5.77. From pH 5.76 to 5.73 both acid and basic dyes passed the barrier equally well. Below pH 5.72 basic dyes penetrated the barrier, whereas acid dyes were completely or almost completely restricted. As also shown in Table IV, the same type of restriction by the barrier was noted when the dye was introduced into the stroma, ruling out the possibility that these observations on the effect of pH refer to substances other than the barrier.

6. Effect of Oxidation on the Stromal-Epithelial Barrier; Determination of E'_0 of Its Oxidation-Reduction System—The effect of oxidation on the barrier was studied by the change it produced in the charge (shift of isoelectric point) on the barrier. Most of the experiments were performed with the supravital technique as follows: Three pieces of tissue were used in each experiment. One as a control was immersed in buffer alone; to the second and third buffer plus oxidant was added; after 5 minutes buffer plus oxidant on the second was replaced by buffer alone and on the third, by buffer plus reducing agent. After another 5 minutes the reducing agent was replaced by buffer alone. The tissue was then stained as usual with the supravital technique. In a few experiments the stroma was filled with dye by intravascular injection under conditions which left the epithelium unstained (see Section 3 for acid dyes and Section 5 for basic dyes). With acid dyes three pieces of tissue were treated as just described with the supravital technique. With basic dyes only buffer and buffer plus oxidant could be used. The dyes of choice were crystal violet (basic) and rose bengal (acid).

The supravital and intravascular experiments gave the same results. In Table V at pH 5.85 it may be seen that all oxidants used reversed the selective permeability of the barrier to acid and

basic dyes and that the normal selective permeability could be restored by reduction with hydrosulfite. Table V also shows that the isoelectric point of the oxidized barrier was approximately at pH 6.5, and that the isoelectric zone covered between 0.57 and 0.73 pH units.

The E_h of the reversible oxidation-reduction system of the barrier at which the charge on the barrier was reversed, as shown

TABLE V
*Shift of Isoelectric Point of Stromal-Epithelial Barrier by Oxidation.
Determination of E'_0 of Oxidation-Reduction System of
Stromal-Epithelial Barrier*

+ represents penetration and 0 no penetration through the stromal-epithelial barrier.

pH	Reducing agent*	Normal		Quinhydrone		Ferrieyanide		Iodine	
		Acid dyes	Basic dyes	Acid dyes	Basic dyes	Acid dyes	Basic dyes	Acid dyes	Basic dyes
5.85†	0	+	0	0	+	0	+	0	+
5.85†	Hydro-sulfite			+	0	+	0	+	0
6.08	0	+	0	0	+				
6.15	0	+	0	+	+				
6.72	0	+	0	+	+				
6.81	0	+	0	+	0				
6.62	0		0		+				
6.62	Hydroquinone, saturated + quinhydrone, saturated ($E_h = 0.215$)				+				
6.62	Hydroquinone, saturated ($E_h = 0.185$)				0				

* Reducing agent used to reduce barrier after oxidation.

† Acetic acid-acetate buffer; others pyridine-pyridine acetate.

in Table V, was determined at pH 6.62. At this pH quinhydrone reversed the charge on the barrier. A saturated solution of hydroquinone at this pH reduced the barrier sufficiently to restore the normal selective permeability; whereas a solution saturated with hydroquinone and quinhydrone failed to do so. This placed the E_h of the barrier system between +0.185 (minimum measured potential of saturated hydroquinone) and +0.215 volt at pH 6.62. About the same value of E_h was found when mixtures of oxidant and reductant of 2,6-dichlorophenol indophenol were used.

An equimolecular mixture of oxidant and reductant reversed the charge on the barrier. The normal charge was restored by a reduced solution of this indicator containing only a trace of the oxidant. Since the ratio of oxidant to reductant of this indophenol does not remain fixed, the potential values obtained with the quinhydrone-hydroquinone system, in which the solid phases were present, are more reliable.

7. *Effect of Calcium Ion on Barrier*—In a series of experiments with the supravital technique it was noted that the stromal-epithelial barrier was considerably more permeable when placed in isotonic NaCl than when placed in Ringer's solution. In isotonic NaCl, crystal violet slowly penetrated from epithelium to stroma, whereas this effect was not noted in Ringer's solution. Under asphyxia, in isotonic NaCl, this dye reached a concentration in the stroma equal to that in the epithelium in 15 to 30 seconds. In Ringer's solution this result took about 5 minutes. At pH 5.8 the barrier normally was impermeable to basic dyes; however, in isotonic NaCl basic dyes slowly diffused through it, making difficult the determination of the isoelectric point. The normal behavior of the barrier was restored if traces of Ca^{++} were added to the isotonic NaCl. Other inorganic ions normally present in plasma were without effect and could not be substituted for calcium. The Ringer's solution was one used by Gey and Gey (8) for tissue culture and had the following composition in gm. per liter: NaCl 8.0, KCl 0.37, CaCl_2 (anhydrous) 0.17, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.21, Na_2HPO_4 0.18, NaHCO_3 0.25. CO_2 gas was added until all components had dissolved.

DISCUSSION

The potentials of +0.100 volt in the epithelium and of -0.130 volt in the stroma of the chorioid plexus, measured by oxidation-reduction indicators in air, must not be considered due to thermodynamic equilibria. Instead they are steady states determined by a balance between the rates of oxidation and reduction. The potential levels of these steady states are the same as the oxidation-reduction potentials of the naturally occurring mediators (reversible, electromotively active oxidation-reduction systems). The potential (ratio of oxidant to reductant) of the mediator is determined by the activities of oxidases and dehydrogenases, O_2 pres-

sure, and the concentration and nature of the substrates. Thus in the presence of indophenol oxidase, the rate of oxidation of the mediator is increased, causing an increase in the ratio of oxidant to reductant and hence an elevation in the potential level of the tissue. The limitation of indophenol oxidase to the epithelium can adequately explain the higher potential of the epithelium.

As is well known, indophenol oxidase (Warburg's iron catalyst) is completely inhibited by cyanide. In this condition the rate of oxidation is determined by other oxidases, presumably the naturally occurring mediators; *e.g.*, Warburg and Christian's flavoprotein. With the inactivation of indophenol oxidase, the rate of oxidation is decreased, causing the potential level of the epithelium to fall to -0.200 volt. The potential of the stroma falls slightly, since it can no longer be oxidized by the epithelium through the reversible oxidation-reduction system of the stromal-epithelial barrier, as noted below. Since the potentials of epithelium and stroma in cyanide are the same, it follows that the ratios of the rates of oxidation due to oxidases not poisoned by cyanide and the rate of reduction by dehydrogenases are equal in the two tissues.

A true thermodynamic equilibrium is measured under N_2 , when the rate of oxidation becomes zero. In N_2 the potentials of epithelium and stroma are the same (-0.290 volt). This may result either from an identity in the substrate-dehydrogenase systems or from an equalization of the two potentials through the oxidation-reduction system of the barrier (see below).

An essential theoretical conclusion which arises from the experimental data is that an electric current exists between epithelium and stroma. The source of the electromotive force for this current is the difference in potential levels of these tissues. Since the potential level of the stroma is lower than that of the epithelium, it will tend to give up electrons to the epithelium (Fig. 2). Evidence, to be discussed presently, indicates that these electrons can pass from stroma to epithelium by means of a reversible oxidation-reduction system contained in the stromal-epithelial barrier. To maintain electroneutrality, cations (basic dyes) must move from stroma to epithelium or anions (acid dyes) in the reverse direction. The selective transfer in air of basic dyes from stroma to epithelium and acid dyes in the reverse direc-

tion, found experimentally, is consequently as would be predicted from the postulated electric current.

Under conditions (cyanide, N_2 , and acid pH) which cause disappearance of the potential difference between epithelium and stroma, the electric current, as indicated by the transfer of dyes, also disappears.

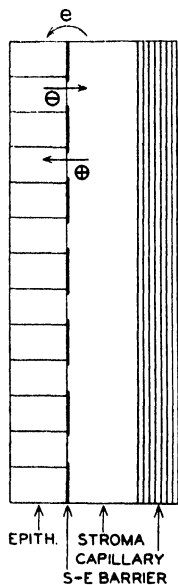


FIG. 2. Schematic representation of the electric current between stroma and epithelium. Electrons from the stroma, which has a relatively low potential, pass to the epithelium by way of the reversible oxidation-reduction system of the stromal-epithelial barrier. To maintain electroneutrality cations must move from stroma to epithelium or anions in the reverse direction.

The results show that the stromal-epithelial barrier contains a reversible oxidation-reduction system. The electron transfer from stroma to epithelium necessary to complete the electrical circuit can occur through this reversible oxidation-reduction system provided this system can be oxidized, at least partially, by the epithelium and reduced by the stroma. To demonstrate this the potential of the stromal-epithelial barrier (E_A) at some known

degree of oxidation (α) of the barrier must be known. This would mean that in the equation $E_h = E'_0 + (RT/NF)\ln(\alpha/(1-\alpha))$, all quantities would be known for calculation of E'_0 . The use of this equation implies that the oxidation-reduction system in the stromal-epithelial barrier can be treated as similar systems in aqueous solution and that neither oxidant nor reductant is associated. The validity of these assumptions must remain questionable.

These measurements cannot be made at physiologic pH but can be determined with basic dyes in the pH region between the upper limits of the isoelectric zones of the reduced and oxidized stromal-epithelial barrier (pH 5.77 and 6.81 respectively). These limits are hereafter referred to respectively as pI_r and pI_o. E_h at a particular pH in this region can be determined by noting the potential necessary to reverse the charge on the barrier. The E_h of the stromal-epithelial barrier of the chorioid plexus at pH 6.62 lies between +0.185 and +0.215 volt.

One of the quantities necessary (7) for the calculation of α is the ratio between the slopes of the pH-titration curves of the reduced and oxidized stromal-epithelial barrier (K_r/K_o). These slopes are proportional to the widths of the isoelectric zones, as is evident from the following consideration. A certain charge density on the barrier is necessary to prevent the passage of oppositely charged ions. It is assumed that this minimal charge density which gives the limits of the isoelectric zone is the same for any state of oxidation of the barrier. Since the pH-charge density curve is directly proportional to the pH-titration curve, the ratio of the widths of the isoelectric zones equals the ratio of the slopes of the titration curves. In the case of the stromal-epithelial barrier of the plexus the isoelectric zone of the reduced barrier is between 0.03 and 0.05 pH unit, while that of the oxidized barrier is between 0.57 and 0.73 pH unit. The ratio of the slopes of the pH-titration curves, therefore, must be between 11 and 25 (0.57/0.05 and 0.73/0.03). The increase in the width of the isoelectric zone noted on oxidation is not unusual, for the dissociation constants of oxidants are generally stronger than those of reductants.

The other quantity necessary to calculate α can be determined from the difference between pI_r and pI_o and the chosen pH at

which E_h is determined. This quantity is equal to the ratio $(\text{pH} - \text{pI}_r)/(\text{pI}_o - \text{pI}_r)$. From the relation

$$\frac{\text{pH} - \text{pI}_r}{\text{pI}_o - \text{pI}_r} = \frac{\alpha}{\alpha + (1 - \alpha)(K_r/K_o)}$$

derived by Friedenwald and Stiehler (7), α can be calculated. For the stromal-epithelial barrier of the plexus at pH 6.62, α lies between 0.98 and 0.995. Assuming a 2 electron system the E'_0 of the barrier at this pH is consequently 0.140 ± 0.025 volt.

To obtain E'_0 at pH 7.4 it is necessary to know the slope of the E'_0 -pH curve. This information is not available, but it is unlikely that it is greater than 0.06. If the assumption be made that the slope is 0.03, E'_0 at pH 7.4 will be $+0.115$ volt. The potential will be shifted by $+0.025$ volt if the slope is 0.0 and by -0.025 volt if the slope is 0.06. E'_0 at pH 7.4 will consequently be $+0.115 \pm 0.050$ volt. If a 1 electron system had been assumed, the E'_0 at pH 7.4 would be $+0.030 \pm 0.085$ volt. In any case it can be seen that the epithelium will tend to oxidize the stromal-epithelial barrier partially and the stroma will tend to reduce it. Therefore the reversible oxidation-reduction system of the stromal-epithelial barrier can transfer electrons from stroma to epithelium.

SUMMARY

1. The potential of the stroma of the chorioid plexus has been found to be -0.130 volt; of the epithelium, $+0.100$ volt. These potentials are correlated with the inequality of distribution of indophenol oxidase between these two tissues. The potentials of epithelium and stroma have been found equal under cyanide anoxia and N_2 asphyxia and are respectively -0.200 volt and -0.290 volt.

2. Basic dyes are selectively transferred from stroma to epithelium and acid dyes in the reverse direction. With cyanide, N_2 , or pH asphyxia this selective transference is abolished.

3. The barrier, demonstrated between epithelium and stroma, is amphoteric with an isoelectric point at pH 5.74. This isoelectric point may be reversibly shifted to about pH 6.4 by oxidation. The potential of the oxidation-reduction system of the barrier at pH 7.4 has been estimated to be $+0.115$ volt. A deficiency of calcium ions was found to increase the permeability of this barrier.

The data are explained by the hypothesis that the difference in potential between epithelium and stroma gives rise to an electric current causing cations to move from stroma to epithelium and anions in the reverse direction. The electrons for this current are carried presumably by the reversible oxidation-reduction system of the barrier between stroma and epithelium. The current disappears when the potential difference disappears.

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BIOCHEMICAL CHANGES ASSOCIATED WITH THE ONSET OF SECRETION IN THE FETAL CHORIOID PLEXUS. AN ORGANIZATION OF OXIDATION-REDUCTION PROCESSES*

BY LOUIS B. FLEXNER AND ROBERT D. STIEHLER

(From the Departments of Anatomy and Ophthalmology, the Johns Hopkins University, Baltimore)

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It has been shown (1) that at the end of the first third of the gestation period of the fetal pig the cerebrospinal fluid changes from an ultrafiltrate to a secretion. The present study is chiefly concerned with the biochemical changes which occur in the chorioid plexus at this transition period, when this organ changes from a passive state to an active chemical machine. This investigation involves primarily a study of the development in the fetus of the secretory mechanism previously described (5) for the functional chorioid plexus.

Material and Methods

Embryo and fetal pigs were obtained under conditions previously noted (2). The chorioid plexuses of embryo pigs with crown-rump lengths between 2.5 and 4.5 cm., intrauterine age 30 to 40 days (6), were used for the presecretory stage; for the secretory stage, plexuses from fetal pigs with crown-rump lengths above 6.0 cm., intrauterine age 43 days, were used. The methods used for the determinations of indophenol oxidase and of oxidation-reduction potentials and for the introduction of dyes into epithelium or stroma have previously been given (3, 5) together with the criteria for the selection of the dyes studied.

Distribution of Indophenol Oxidase—In the presecretory plexus indophenol oxidase was present in equal concentration in epi-

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thelium and stroma. In the secretory plexus this oxidase was absent from the stroma and was present in the epithelium in many times the concentration found in the presecretory plexus.

Oxidation-Reduction Potentials—As shown in Table I, in the pre-secretory plexus, the potential levels in epithelium and stroma, measured by oxidation-reduction indicators, were identical. This

TABLE I

Oxidation-Reduction Potentials of Presecretory and Secretory Fetal Chorioid Plexus in Air

0 represents no reduction, + one-third reduced, ++ one-half to two-thirds reduced, +++ three-fourths to nine-tenths reduced, ++++ over nine-tenths reduced.

Indicator	<i>E'</i> at pH 7.4 volt	Reduction of indicator in			
		Presecretory stage		Secretory stage	
		Stroma	Epithelium	Stroma	Epithelium
Toluylene blue	+0.101	++++	++++	++++	++
Lauth's violet	+0.050	++	++	++++	0
Cresyl blue	+0.034	+	+	++++	0
Methylene blue	-0.002	0	0	++++	0
Indigotrisulfonate	-0.099	0	0	+++	0
Indigodisulfonate	-0.143	0	0	+	0
Cresyl violet	-0.175*	0	0	0	0

Under anaerobiosis

Dimethyl phenosafranine . . .	-0.275	+++	+++	+++	+++
Safranin T	-0.298	++?	++?	+	+

* This potential is in doubt since the cresyl violet sold by the National Aniline and Chemical Company, Inc., is probably different chemically from the cresyl violet measured by Rapkine, Struyk, and Wurmser (4).

potential was about +0.045 volt at physiologic pH. In the secretory plexus, from the last third of the gestation period, the potential in the epithelium was +0.100 volt and in the stroma was -0.130 volt. In the earliest secretory plexus (crown-rump lengths of 6.0 to 8.0 cm.), the potential in the epithelium was about +0.060 volt and in the stroma -0.040 volt. With increase in age of the plexus, the potentials of epithelium and stroma gradu-

ally approached those noted above for the last third of the gestation period. Under N_2 asphyxia (Table I) the potentials of epithelium and stroma of both presecretory and secretory plexuses were practically equal and had the value -0.290 volt.

Passage of Dyes Through Stromal-Epithelial Barrier—The passage of dyes from epithelium to stroma was studied by the supravital technique; and from stroma to epithelium by intravascular injection (3, 5). As shown in Table II, with the presecretory plexus, acid, basic, and neutral dyes passed equally well from epithelium to stroma and from stroma to epithelium. In the

TABLE II

Passage of Dyes Through Stromal-Epithelial Barrier of Presecretory and Secretory Fetal Chorioid Plexus at Physiologic pH

0 represents no penetration, + slow diffusion, ++ rapid diffusion, ++++ accumulation beyond diffusion.

Dye	Ionic type	Penetration			
		Presecretory stage		Secretory stage	
		Epithelium to stroma	Stroma to epithelium	Epithelium to stroma	Stroma to epithelium
Crystal violet.....	Basic	+	+	0	++++
Malachite green.....	"	+	+	0	++++
Cresyl violet.....	"	+	+	0	++++
Eosin.....	Acid	++	++	++	Trace
Rose bengal.....	"	++	++	++	"
Brom-phenol blue.....	"	++	++	++	"
Rhodamine B.....	Neutral	++	++	++	++

secretory stage, acid dyes passed from epithelium to stroma but not in the reverse direction, while basic dyes accumulated in the epithelium from the stroma but did not pass into the stroma from the epithelium. Neutral dyes passed equally well in both directions.

In the presecretory plexus the passage of dyes in either direction was not affected by cyanide or N_2 asphyxia. The secretory plexus under asphyxia behaved like the presecretory plexus except as follows: Dyes (fast green, methylene blue), which did not pass readily from stroma to epithelium in the earliest secretory stage, passed into the epithelium in the presecretory stage. This was

taken as evidence that the stromal-epithelial barrier of the pre-secretory plexus was more permeable than that of the secretory plexus. Results of the same kind were obtained in experiments to determine the isoelectric point of the stromal-epithelial barrier of the presecretory plexus. As has been recorded (5), the isoelectric point of the stromal-epithelial barrier of the secretory plexus lies at pH 5.74. With the secretory plexus, basic dyes penetrated at pH values lower than 5.74 and were restricted at higher pH values. The reverse was true for acid dyes. The experimental observations with the presecretory plexus were difficult, since acid and basic dyes penetrated on both sides of pH 5.74 and the isoelectric point of the stromal-epithelial barrier had to be judged by the effects of pH on the rates of penetration of these dyes. In a few favorable experiments, however, the behavior of acid and basic dyes indicated that the isoelectric point of the stromal-epithelial barrier of the presecretory plexus was the same as that of the secretory plexus.

Passage of Reduced and Oxidized Indicators; Reducing Ability of Stroma—Methylene blue and safranin in 0.01 M solutions were introduced into the stroma by intravascular injections (3, 5) both in the oxidized and reduced states. The reduced indicators were prepared with a slight excess of hydrosulfite so as to keep the indicator reduced in the stroma. When the ventricles were irrigated with Ringer's solution, to avoid possible anoxia, the same results were obtained as without irrigation. The observations recorded in Table III were made within 5 minutes after injection of the indicator; with time, the distribution of dye was the same in the young and old secretory plexuses.

As shown in Table III, leucodimethyl phenosafranin, in both the young and old secretory plexus, passed rapidly from stroma to epithelium, where it was oxidized. The oxidant of this indicator, however, which was not reduced in air by the stroma of even the old secretory plexus, passed extremely slowly into the epithelium. Leucomethylene blue behaved like the leucosafranin. Methylene blue in the young secretory plexus passed very slowly into the epithelium, as did safranin, and even more slowly than in the pre-secretory plexus. In the old secretory plexus, however, methylene blue passed as rapidly into the epithelium as did leucomethylene blue.

Histological Observations—In the fresh preparation the following histological differences were noted between the presecretory and secretory plexus: (1) The epithelium of the presecretory stage was higher than that of the secretory stage. With crown-rump lengths between 3.0 and 4.0 cm. the epithelial height varied between 20 and 26 μ ; between 6.0 and 9.0 cm., the height was from 10 to 14 μ ; above 20.0 cm., the height was from 8 to 12 μ . (2) The secretory plexus was more tufted than the presecretory. (3) The distance between the stromal-epithelial barrier and the capillaries was greater in the presecretory plexus. (4) The secretory plexus was more vascular.

TABLE III

Effect of Age of Plexus on Passage of Oxidation-Reduction Indicators from Stroma to Epithelium

+ represents slow passage into epithelium with accumulation, ++ passage into epithelium without accumulation, ++++ rapid passage into epithelium with accumulation.

Indicator	Passage of indicator in fetus, crown-rump length		
	3.0-4.0 cm.	6.0-9.0 cm.	About 20 cm.
Methylene blue oxidant.....	++	+	++++
“ “ reductant.....	++	++++	++++
Dimethyl phenosafranine oxidant.....	++	+	+
“ “ reductant.....	++	++++	++++

Preparations fixed in formol-Zenker's solution and stained with hematoxylin and eosin showed these additional differences between the two stages of the plexus: (1) The cytoplasm of the epithelial cells of the presecretory plexus stained more brilliantly with eosin than those of the secretory plexus. (2) The nuclear chromatin content of the presecretory epithelium was less than that of the secretory stage. No change in mitochondria was noted with onset of secretion.

Growing Tip of Plexus—That part of the plexus which lies in the lateral ventricle most distant from the foramen of Monro is the actively growing tip of the plexus. In all the respects noted above, this growing tip behaved like the presecretory plexus.

DISCUSSION

In a previous paper (5) a secretory mechanism of the chorioid plexus, by which energy from oxidation-reduction processes may be converted into work, has been described. In brief, this secretory mechanism is an electric current which causes cations to move from stroma to epithelium and anions in the reverse direction; the source of E.M.F. for this current is the difference in potential of stroma and epithelium; the electrons for this current are carried by the reversible oxidation-reduction system of the stromal-epithelial barrier.

The present paper is concerned with the changes in the fetal chorioid plexus which occur with the onset of secretion, as judged by changes in the distribution ratios of diffusible substances in cerebrospinal fluid and blood plasma (1). The onset of secretion, judged in this way, is accompanied by the establishment of an electric current. The evidence for this is as follows: Prior to secretion, indophenol oxidase is equally distributed between epithelium and stroma; the potential levels of epithelium and stroma are the same; and there is no favored direction for the transference of acid and basic dyes across the stromal-epithelial barrier. With onset of secretion, indophenol oxidase is confined entirely to the epithelium and is in greater concentration than in the presecretory epithelium; a difference in potential develops between epithelium and stroma; and basic dyes pass only from stroma to epithelium and acid dyes only in the reverse direction. The transference of dyes in the secretory plexus under asphyxia is like that in the presecretory plexus in air. Asphyxia has no effect on the transference of dyes in the presecretory plexus.

The potential levels in air can be correlated with the distribution and concentration of indophenol oxidase, as previously noted (5). With the onset of secretion, the potential of the epithelium rises slightly and the potential of the stroma falls markedly. As the plexus becomes older, the potential of the epithelium continues to rise and the potential of the stroma to fall. With the onset of secretion the potential difference between epithelium and stroma changes from 0 to about 0.100 volt; this potential difference increases to about 0.230 volt in the old secretory plexus.

Associated with the potential decrease in the stroma there is an

increase in its reducing ability. Indicators such as dimethyl phenosafranin, which are not reduced by the stroma in air, pass slowly into the epithelium. However, methylene blue which is reduced by the stroma in air passes very rapidly into the epithelium in the old secretory plexus. In the young secretory plexus, methylene blue passes slowly into the epithelium, like the safranines. Both these indicators pass rapidly into the epithelium when injected in the reduced state. From this it is inferred that it is the reduced indicator which passes rapidly into the epithelium and that the stroma of the old secretory plexus reduces methylene blue much more rapidly than the stroma of the young secretory plexus. Since it is reasonable to assume that substrates of young and old secretory plexus are in equal concentrations, the increased reducing ability of the old plexus is believed to be due to an increase in the concentration or activity of the dehydrogenases. Methylene blue and the safranines accumulate in the epithelium as do other basic dyes, *e.g.* crystal violet, but do so more slowly presumably owing to their larger molecular volume (3). Neutral, reduced indicators pass into the epithelium and accumulate as the oxidant, since they are oxidized at the potential of the epithelium. In the presecretory plexus, these indicators cannot be reduced by the stroma and so pass into the epithelium as the oxidants. Their rates of penetration agree with other evidence already noted that the stromal-epithelial barrier of the presecretory plexus is more permeable than that of the secretory plexus.

SUMMARY

The onset of secretory activity in the fetal chorioid plexus is associated with the following changes.

1. In the presecretory plexus indophenol oxidase is in equal concentration in epithelium and stroma. In the secretory plexus this oxidase is limited to the epithelium and is in greater concentration.

2. In the presecretory plexus there is no potential difference between epithelium and stroma. With onset of secretion, a potential difference of 0.100 volt develops which increases with age to 0.230 volt.

3. In the presecretory plexus there is no selective transference

of dyes. In the secretory plexus, the chosen basic dyes pass only from stroma to epithelium and the chosen acid dyes in the reverse direction.

4. The reducing ability (dehydrogenases) of the stroma increases with age.

5. Certain histological differences between the presecretory and secretory plexuses have been noted.

The changes which occur with the onset of secretion are correlated with the theory that the electric current, which develops between epithelium and stroma at this time, is associated with the secretory process.

It is a pleasure to acknowledge our gratitude to the Wm. Schlumberger-T. J. Kurdle Company for the fetal material.

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SOME OBSERVATIONS ON HYPAPHORINE: RACEMIZATION OF ITS ESTER AND PROPERTIES OF OTHER DERIVATIVES

BY WILLIAM M. CAHILL AND RICHARD W. JACKSON

(From the Department of Biochemistry, Cornell University Medical College, New York City)

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The procedure of van Romburgh and Barger (1) for the synthesis of the betaine, hypaphorine, entails the methylation of *l*-tryptophane by boiling for several hours a solution of the amino acid in methyl iodide and methanol kept slightly alkaline with sodium hydroxide. The resulting betaine methyl ester iodide is then subjected to a very brief hydrolysis in hot 1 per cent sodium hydroxide, and the betaine liberated as the free base is recovered by precipitation with dilute nitric acid. The insoluble salt secured may be converted (Barger (2)) to the betaine by treatment with a concentrated solution of sodium carbonate. No loss of optical activity was presumed to be induced by this method of synthesis.

We discovered, however, in repeating these reactions (3) that recrystallized specimens of the methyl ester iodide obtained in the above manner from tryptophane, abrine, or hypaphorine possessed divergent specific rotations ($4-9^\circ$ in methanol). The preparations were suspected, therefore, of being partially racemized. Moreover, the betaines derived from them always exhibited a specific rotation less than that of $+113.5^\circ$ for hypaphorine (3), and in one instance the product was devoid of optical activity. It appeared desirable, therefore, to prepare a methyl ester iodide of the betaine of tryptophane of considerably higher optical activity, and then to submit it to the action of alkali as employed in each of the two likely racemizing steps of the stated procedure for the synthesis of hypaphorine. The preparation of a methyl ester iodide with a specific rotation of $+48.8^\circ$ (about 39° in methanol)

was accomplished by simply boiling a solution of hypaphorine in methanol and methyl iodide. When this active betaine ester was heated with sodium hydroxide either in methanol or in water, under conditions prevailing in the synthesis of the tryptophane betaine, the product was 90 to 100 per cent racemized. Nevertheless, the van Romburgh-Barger method does give a limited yield of the optically active betaine which may be isolated from the mother liquors of the largely racemized betaine ester (3). It seems probable that this optically active fraction escapes racemization by not undergoing esterification.

The lability of tryptophane betaine ester to racemization in the presence of alkali is analogous to that of alanine betaine ester studied by Fischer (4) and further investigated by Biilmann and associates (5, 6). These findings are of possible interest with regard to the preparation of racemic betaines. The device often employed for the racemization of amino acids, *viz.*, heating with rather concentrated caustic alkali solution, causes the corresponding betaines to undergo degradation. Consequently, the ease with which the esters of hypaphorine and alanine betaine sustain loss of optical activity upon mild treatment with alkali indicates a convenient method for preparing the racemic form of a betaine from one of the enantiomorphs. On the other hand, this tendency may be expected to interfere in the conversion of optically active amino acids to optically active betaines when the procedure involves exposure of the betaine ester to alkali. This is the case with the Engeland (7) methylation reaction applied by van Romburgh and Barger for the synthesis of hypaphorine.

A study was also made of the nitrate and iodide salts of hypaphorine. Van Romburgh and Barger (1) state that the nitrate salt of hypaphorine may be prepared by hydrolyzing the methyl ester iodide in alkali and acidifying with dilute nitric acid. We have found that the precipitation of the base in this manner from a solution containing appreciable iodide ion invariably yields a product contaminated with iodine. Failure to free the salt from the impurity by recrystallization from water or from ethanol led us to the discovery of an insoluble hypaphorine iodide. When a great deal of iodide ion is present, as is the case with the mother liquors from the hypaphorine methyl ester iodide synthesized with the aid of methyl iodide, the salt precipitated with nitric acid may

consist entirely of the iodide. Our reference specimens of the nitrate and iodide were prepared by the addition of dilute nitric or hydriodic acid to an aqueous solution of authentic hypaphorine. The two salts are both quite insoluble and melt with decomposition at nearly the same temperature, but yield excellent differentiating analyses according to their respective postulated formulas.

EXPERIMENTAL

Preparation of Hypaphorine Methyl Ester Iodide of High Rotation—0.92 gm. of hypaphorine ($[\alpha]_D^{25} = +113.6^\circ$) was refluxed for $7\frac{1}{2}$ hours in a mixture of 45 cc. each of methanol and methyl iodide, both carefully dried and redistilled. The mixture was evaporated *in vacuo* to dryness, and the residue, crystallized from water, amounted to 0.87 gm. The melting point of $202\text{--}203^\circ$ and $[\alpha]_D^{25} = +48.8^\circ$ (42.7 mg. dissolved in 5 cc. of water) did not change after the second recrystallization. Analysis gave results as follows: for $C_{15}H_{21}O_2N_2I$, calculated, C 46.38, H 5.45, I 32.71; found, C 46.30, H 5.56, I 32.52. The melting point of this optically active methyl ester iodide which crystallizes as needles is 8° higher than that of the crystalline racemic substance which was observed in the form of thick plates (3). Van Romburgh and Barger's preparation of the methyl ester iodide consisted of glistening plates melting at 197° (1).

Racemization Experiments on Hypaphorine Ester—94 mg. of the methyl ester iodide ($[\alpha]_D^{25} = +48.8^\circ$) were refluxed for 8 hours in a mixture of 1.25 cc. each of methanol and methyl iodide with 10 mg. of sodium hydroxide. 30 mg. of sodium hydroxide dissolved in 0.7 cc. of methanol were added in 2.1 mg. portions every half hour during the first 7 hours. The mixture was concentrated to less than 1 cc. and allowed to crystallize. 89 mg. of ester iodide were recovered. The optical rotation was 0° .

40.2 mg. of active methyl ester iodide were heated in 2 cc. of 1 per cent aqueous sodium hydroxide for 2 minutes in a boiling water bath. The solution was immediately cooled, diluted to 5 cc., and the optical activity determined. $[\alpha]_D^{25} = +10.7^\circ$, calculated for the free betaine produced in the hydrolysis. Thus the resulting hypaphorine was more than 90 per cent racemized.

¹ All melting points are corrected.

When the unesterified hypaphorine (employed as the iodide salt) was treated with alkali in exactly the same manner, the specific rotation remained unaltered ($[\alpha]_D^{25} = +113.7^\circ$). It was established that the specific rotation of hypaphorine is the same whether the solvent is water or the 0.4 per cent sodium hydroxide solution as used in these experiments.

*Racemic Hypaphorine*²—The optically inactive betaine was prepared from racemized methyl ester iodide and was twice crystallized from 95 per cent ethanol. The product consisted of rhomboidal plates, melted with decomposition at 248–249°, and was totally devoid of optical activity. Following air drying, it was found to contain 15.3 per cent of volatile substance, whereas 2 moles of water would account for a 12.8 per cent loss in weight. The crystals lose their sheen upon dehydration. In contrast, the optically active betaine crystallized from 95 per cent ethanol and air-dried is practically anhydrous. Barger (2) reports 2 moles of water of hydration for a preparation of the betaine. The N calculated is 11.38; found for our dehydrated racemic preparation, 11.26.

Hypaphorine Nitrate and Iodide—Pure hypaphorine nitrate was prepared by dissolving 0.5 gm. of hypaphorine ($[\alpha]_D^{25} = +113.4^\circ$) in 2 cc. of water and adding dilute nitric acid. The product filtered off, washed with water, and carefully dried *in vacuo* amounted to 0.49 gm. The crystals consisting of short prisms melted with decomposition at 217–219°. 58.3 mg. dissolved in 5 cc. of water containing 0.1 cc. of 15 N ammonium hydroxide gave $[\alpha]_D^{25} = +91.2^\circ$, or $+114.5^\circ$, calculated for hypaphorine. The ammonia used to dissolve the insoluble nitrate was shown to have no appreciable effect on the optical rotation of hypaphorine base under comparable conditions. For $C_{14}H_{18}O_2N_2 \cdot HNO_3$, the calculated values for C and H are 54.33 and 6.19; the values found were 54.54 and 6.25. Van Romburgh and Barger's preparation of the nitrate melted at 215–220° and gave $[\alpha]_D = +75.4^\circ$, or $+94.7^\circ$, calculated for hypaphorine.

² Some years ago it was shown (8) that the betaine of tryptophane cannot replace tryptophane in the diet for the promotion of growth. Inasmuch as the optical activity of the betaine specimen employed was not then determined, we have reexamined the substance. It is the pure racemic form.

The iodide was secured by treating 225 mg. of the authentic hypaphorine dissolved in 9 cc. of water with 0.13 cc. of freshly distilled 55 per cent hydriodic acid. The mixture was evaporated to dryness over sodium hydroxide *in vacuo*, and recrystallized from water to give wedge-shaped prisms melting with decomposition at 220–221°. The yield was 233 mg. 49.0 mg. dissolved in 5 cc. of water containing 0.1 cc. of ammonium hydroxide gave $[\alpha]_D^{25} = +75.2^\circ$, or $+114.3^\circ$, calculated for the betaine itself. The calculated I for $C_{14}H_{18}O_2N_2 \cdot HI$ is 33.93; the value found was 33.70.

SUMMARY

The methyl ester (iodide) of hypaphorine is shown to be easily racemized in alkali.

The preparation of hypaphorine nitrate is discussed, and another insoluble hypaphorine salt, the iodide, described. Their specific rotations are in accord with the value of $+113.5^\circ \pm 1^\circ$ for the betaine itself.

The racemic betaine of tryptophane is described.

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THE INFLUENCE OF ASPHYXIA AND OTHER FACTORS ON THE SERUM POTASSIUM OF CATS

BY MCKEEN CATTELL AND HELEN CIVIN

*(From the Department of Pharmacology, Cornell University Medical
College, New York City)*

(Received for publication, August 1, 1938)

The importance of potassium in the maintenance of normal physiological function and life in the cell has long been recognized. The fact that osmotic equilibrium with the extracellular fluids is attained through a relatively high concentration of potassium in the interior of the cell, and that this condition is essential to the maintenance of excitability and other properties of the cell upon which life depends, emphasizes the rôle of potassium in the economy of the organism. The problem has been the subject of a large number of investigations from various points of view, and the literature cannot be covered here. The recent work of Fenn and his associates has given much information regarding the conditions of electrolytic equilibrium in cells, and the factors which modify it, with special reference to muscle. This work as well as the pertinent earlier literature is summarized in reviews by Fenn (1936).

Until recently very little attention had been given to the question of potassium metabolism in the intact animal. The equilibrium between the blood and the cells, the factors which cause the passage of potassium to and from the cells, and the significance of these shifts are matters of fundamental importance, which are now receiving the attention of workers in a number of laboratories. Our interest in the problem arose in connection with a study of the influence of the digitalis glucosides on the potassium metabolism of cats. It was soon discovered that the blood serum of these animals was subject to large and rapid variations of potassium under apparently physiological conditions. It is the purpose of the present paper to describe some of these variations, with special reference to the influence of asphyxia.

Methods

All the observations were made on cats, which usually had been without food for from 24 to 48 hours. In those experiments requiring an anesthetic, 50 mg. per kilo of "dial"¹ were given by the peritoneum.

Blood samples were taken, as required, from the jugular vein, femoral artery, or the heart. From 5 to 6 cc. of blood were withdrawn for each sample; a procedure which was repeated not more than from five to seven times except in animals observed over a period of several days. Hemorrhage is known to cause a rise in blood potassium (Thaler, 1935) but the total amounts withdrawn over a period of several hours is too small to be significant in these animals which weighed from 3.5 to 5.0 kilos. Moreover, in most experiments the results of a procedure were related to a control sample taken only a few minutes before. The blood was drawn directly into or immediately transferred to a centrifuge tube, where it was allowed to clot for from 15 to 30 minutes before the serum was separated.

The potassium determinations were made by the Wenger, Cimerman, and Rzymowska (1936) modification of the Shohl and Bennett method with slight further changes. 1 cc. of serum was ashed in a silica crucible with 5 cc. of a mixture of 5 parts of nitric acid and 2 parts of perchloric acid. If the oxidation of the organic matter was not complete when all the acid had been evaporated over a hot-plate, the crucibles were placed in a muffle furnace at 400–450° and the ashing completed. This rarely took longer than 10 minutes. Any ammonium compounds were decomposed by the addition of a few drops of 20 per cent NaOH and the solution was evaporated to dryness. The residue was then acidified by the addition of a few drops of perchloric acid (a slight excess) and the solution evaporated to dryness. Heating was continued until fuming ceased. The residue was then taken up in a few drops of water and 0.3 cc. of a 10 per cent solution of chloroplatinic acid added. The solution was again evaporated just to dryness and the precipitate washed with 6 cc. of 95 per cent alcohol in successive portions of 0.5 cc. each by use of a microfilter

¹ Diallylbarbituric acid with ethyl carbamate, kindly supplied by Ciba Pharmaceutical Products, Inc.

stick (Jena, No. 91G3). The precipitate was heated at less than 100° until all the alcohol was evaporated and then dissolved in about 2 cc. of CO₂-free water and 1 cc. of a freshly prepared solution of 2 N KI added. The crucibles were returned to the hot-plate, allowed to stand about 4 minutes, and titrated to a clear yellow with freshly standardized 0.01 N sodium thiosulfate. This method has the advantage of requiring no transfers of solutions or precipitate. All determinations were carried out in duplicate.

TABLE I
Serum Potassium in Unanesthetized Cats

Cat No.	Potassium		Interval
	Sample 1	Sample 2	
	mg. per 100 cc.	mg. per 100 cc.	min.
1	20.2		
2	19.7		
7	25.4	27.4	6
8	25.6	30.2	6
9	28.2	22.3	17
11	30.3		
12a	19.3	21.4	15
12b	11.6	10.2	48
13	24.1		
15	23.9	23.9	15
18	17.4		
Average.	22.3		

Results

Normal Variability—The blood potassium has been determined in the course of various experiments in a fairly large group of animals before they were subjected to experimental procedures. We were surprised to find a wide range of potassium values in apparently normal animals. The figures for the first blood sample, and in some cases a second sample, for eleven cats which had been without food for 24 to 48 hours are given in Table I. The blood was obtained from the external jugular vein by venipuncture. Under those conditions the serum potassium ranged from 30.3 to 10.2 mg. per 100 cc., and averaged 22.3 mg. These variations apparently are not related to individual differences in cats, for

equally large changes were observed from day to day in the same animal. Two striking examples, selected for their variability, are charted in Fig. 1. During the period of the experiment the animals were given no food other than milk.

In another series of animals the first blood sample was taken after anesthesia had been produced by the intraperitoneal injection of 50 mg. per kilo of dial. The data for nineteen such experiments are included in the column headed "First control" in Table II, in which the time after the injection of the anesthetic is also indicated. It will be noted that in this series also there is a marked variability in the potassium of the blood, ranging in the nineteen animals from 3.6 to 26.5 mg. per 100 cc. of serum, with

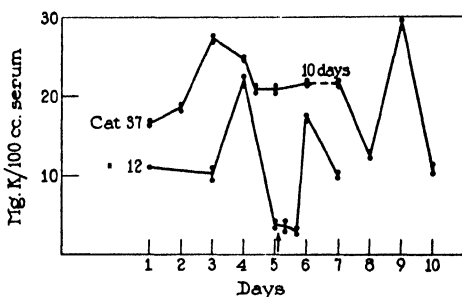


FIG. 1. Variations in serum potassium occurring from day to day in two unanesthetized cats. In this and subsequent charts duplicate determinations are shown for each blood sample. At the arrow 0.05 mg. per kilo of ouabain was injected intravenously in Cat 12.

an average of 17.8 mg. In these experiments successive samples gave relatively constant values during a period of from 4 to 8 hours, except when they were influenced by some experimental procedure. This is illustrated by the final potassium values in Table II (last column) which are generally of the same order as those determined for the first control sample.

Effect of Asphyxia—Because of the very large changes in blood potassium occurring as the result of oxygen lack in the cat, we have carried out a number of experimental procedures designed to throw light on the mechanism of this phenomenon, with special reference to the rôle of respiratory factors. Asphyxial rises in blood potassium of relatively small magnitude have been de-

scribed in dogs (Houssay, Marenzi, and Gerschman, 1936, 1937; Dennis and Mullin, 1938). The incidental observation of a high blood potassium from asphyxia in the cat is mentioned by D'Silva (1934).

TABLE II
Effect of Asphyxia on Serum Potassium

Cat No.	First control after dial		Preasphyxia control		Asphyxia		Recovery	
	min.*	mg. K per 100 cc.	min.*	mg. K per 100 cc.	min.†	mg. K per 100 cc.	min.‡	mg. K per 100 cc.
13	160	18.2	420	17.2	4	47.5		
14	32	22.7	32	22.7	4	28.2	220	20.2
16	15	13.4	76	17.7	7	57.7	29	18.3
17	48	21.2	48	21.2	12	45.7	6	20.9
19	60	6.4	60	6.4	6	26.2	36	11.9
20	185	10.7	375	15.4	12	53.8		
21§	73	21.0	73	21.0	2	44.8		
22	23	24.6	23	24.6		46.8	6	26.5
23§	40	26.5	100	25.3	2½	32.9	10	20.0
24	106	25.0	157	24.0	5½	47.8		
25	400	23.4	470	19.2	5	30.1		
28	90	18.5	128	18.6	9	35.5	10	13.5
30	40	19.6	83	10.4¶	6	41.7		
32	55	19.8	216	18.5¶	5	36.9		
43	37	10.2	37	10.2	4	39.5	9	10.6
45	39	20.6	39	20.6	4	33.8	11	16.7
46	30	3.6	30	3.6	4	18.0	20	4.5
51	40	16.6	40	16.6	4	32.2	16	16.7
53	86	18.0	86	18.0	4½	24.5	18	15.6
Average.....		17.8		17.4		38.1		

* Time elapsing between the injection of dial and taking of the blood sample.

† Time from start of asphyxia.

‡ Time from end of asphyxia.

§ Asphyxia produced by artificial respiration with nitrogen.

|| Previously adrenalectomized.

¶ The cat had been given 0.3 mm per kilo of NaOH intravenously.

When the external respiration is completely arrested by opening the chest wall in anesthetized animals, a marked rise in the blood potassium takes place. In blood samples taken after 4 to 5 minutes of arrested respiration the average potassium content

of the serum was about twice that of the controls. The results of nineteen such experiments are summarized in Table II. The 4 or 5 minute period of asphyxia employed approximates that required to bring about a failure of the circulation. To insure a satisfactory sample of blood under these conditions of low blood pressure, the final sample was usually taken directly from the heart. This degree of asphyxia did not result in permanent damage, and the heart rapidly resumed its normal activity upon re-establishment of the respiration. Individual experiments show a considerable variation in the course of the rise in blood potassium.

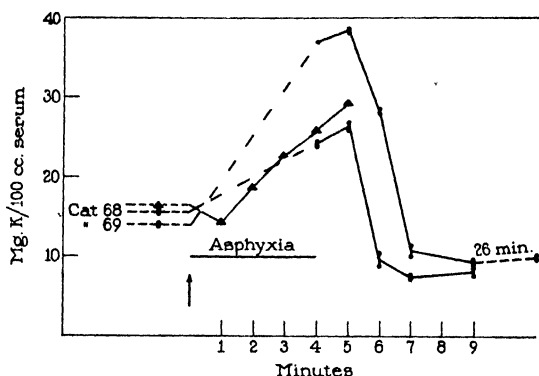


FIG. 2. Experiments showing the course of the blood potassium changes occurring during and following asphyxia. In Cats 68 and 69 the rate of recovery is shown from samples taken at 1 minute intervals. The other curve (points designated with triangles) represents the results from four cats and shows the average serum potassium values at frequent intervals during a 5 minute asphyxial period.

The average results for four cats in which blood samples were secured at intervals of 1 minute during the period of asphyxia are charted in Fig. 2. The drop in potassium during the 1st minute was always present and averaged 2 mg. per 100 cc. of serum. The potassium concentration in the 2 minute sample was significantly higher than the controls and the rise continued, somewhat irregularly in the individual experiments, until respiration was restored at the end of the 5 minute period.

In many experiments the blood potassium was determined again at various intervals after the respiration was reestablished,

and these values are shown in the last column of Table II. They are within the range of the earlier controls, and they show the process to be completely reversible. In two animals detailed studies were made of the rate of disappearance of the extra potassium from the blood stream, and these are charted in Fig. 2. Blood samples were taken at intervals of 1 minute, and they reveal the surprising fact that the potassium concentration returns to normal or falls below normal within a period of 2 minutes. The persistence of low potassium values for a short period after asphyxia has been characteristic of a number of experiments in which analyses were made on blood samples taken during that period.

There are several further points of interest in connection with these results. In the first place, there appears to be a limiting value to the concentration of potassium reached during asphyxia, and values greater than 50 mg. per 100 cc. of serum were rarely obtained. Cats having initially a low potassium concentration usually showed a several fold increase as the result of asphyxia; whereas, when the potassium was already high, the percentage increase was relatively small. Longer periods of asphyxia are not effective in further increasing the blood potassium, presumably because the blood no longer circulates from the capillaries to the heart. However, blood samples obtained immediately after the circulation is reestablished show a slight further elevation in potassium content (see Fig. 2), which in all probability represents potassium accumulated in the blood previously stagnant in the capillary bed.

In order to gain insight into the factors concerned in the asphyxial rise of blood potassium several variations of procedure were instituted. Several animals (*e.g.*, Cat 28, Table II) were adrenalectomized before being subjected to asphyxia, a procedure which did not appear to influence the typical elevation of potassium. In two animals (Cats 21 and 23) an attempt was made to eliminate the factor of an increase in carbon dioxide by means of forced respiration with pure nitrogen. Under these circumstances evidence of asphyxia comes on, as might be expected, with great rapidity. The potassium response, however, appeared not to differ from that occurring as a result of arresting the respiration by opening the chest wall. A few experiments have been carried

out in which the carbon dioxide concentration has been raised in the inspired air by administering a mixture of 10 per cent CO_2 and 90 per cent O_2 . This caused a well marked but temporary rise in the blood potassium, with a return to normal values while the high carbon dioxide was being continued (see Fig. 3). The mechanism of this effect requires further study, but it is not unlikely that it is associated with a transient stimulation of the adrenals, which is known to cause a rise in blood potassium.

Because of the possible importance of the liver as a source of the increased blood potassium (Houssay *et al.*, 1936), several experiments were carried out in eviscerated animals. However, their

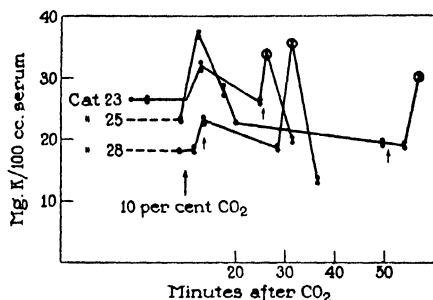


FIG. 3. The effect of breathing a mixture of 10 per cent CO_2 and 90 per cent O_2 on the serum potassium in three cats. Discontinuance of the CO_2 is indicated by a small arrow in each experiment. The encircled points represent the blood serum values at the end of a 5 minute period of asphyxia.

condition was such that we did not consider the results reliable, and for this reason the data have been omitted from the tables. Further experiments of this character are now in progress.

DISCUSSION

The observation that asphyxia causes a rise in blood potassium is not new. Houssay, Marenzi, and Gerschman (1936) have studied the phenomenon in dogs given chloralose. They observed an increase of from 1.2 to 8.6 mg. of potassium per 100 cc. of plasma (average 4.7 mg. per 100 cc. in four animals) in blood samples taken 1 minute after the termination of a period of from $2\frac{1}{2}$ to 4 minutes of complete asphyxia. This asphyxial rise in blood potassium did not occur when both the adrenals and liver were removed, or their nerves sectioned, and the liver

was therefore considered to be the source of the extra potassium. Larger changes are shown in a preliminary report by Dennis and Mullin (1938) which has just appeared: in twelve dogs anesthetized with nembutal the average concentration of potassium in the plasma rose from 18.1 mg. per 100 cc. to 22.2 at the end of a 2 to 4 minute period of asphyxia. In four animals the asphyxia was repeated, resulting in an average rise of from 16.0 to 23.9 mg. per 100 cc. of plasma. The only observation on the effects of asphyxia on the potassium concentration in cats of which we are aware is that of D'Silva (1934) who mentions, without giving data, that acute asphyxia produces a rise in serum potassium.

The present experiments show that cats respond to a 4 to 5 minute period of asphyxia with an extremely marked rise of blood potassium; increases of the order of 2 to 4 times the control value being the rule, and the potassium commonly reaches a maximum value between 40 and 50 mg. per 100 cc. of serum. It is of interest to note that Houssay and Marenzi (1937) found the largest intravenous dose of potassium tolerated by dogs to be 20 mg. per kilo. This is almost the same dose as that observed for cats by Dr. Harry Gold in this laboratory. In a group of experiments the minimum lethal dose of potassium was found to be between 15 and 20 mg. per kilo injected rapidly as potassium chloride. Thus, on the basis of an estimated blood volume equivalent to 10 per cent of the body weight, the maximum potassium concentration in the blood must not have exceeded the values found in many animals as a result of asphyxiation, from which the animal readily recovered.

The return to normal blood potassium values with the resumption of the respiration occurs with remarkable rapidity. Experiments are recorded (Fig. 2) in which as much as 16 mg. per 100 cc. of blood, representing more than half the total potassium contained in the blood, at the given time, disappeared in 1 minute. Under such conditions the potassium falls below its original level, as was observed by Houssay and his associates in dogs, and the changes are similar to those reported by D'Silva (1934) following the injection of epinephrine in cats. The observations of Houssay and Marenzi (1937) and Brewer and Larson (1938) show that injected potassium also disappears from the blood stream with great rapidity.

These sudden large shifts in potassium, together with the wide

variations found among individual cats and in the same animal from day to day, suggest that the mobilization of potassium may represent an important regulatory mechanism. Various suggestions have arisen regarding the possible biological significance of these changes, including (1) an inadequate oxygen supply for the maintenance of the normal function of the cell membrane, thus permitting an escape of potassium from the interior of the cell, and (2) a shift of potassium between the blood and the cells of the body associated with the acid-base requirements of the tissues.

The first suggestion receives some support in the experiments of Baetjer (1935), who found that when the blood supply through the leg muscles of the cat was reduced by 80 per cent or more a marked increase in the plasma potassium occurred in the returning venous blood.² Further, hemorrhage (Thaler, 1935) and the low blood pressure accompanying shock (Zwemer and Scudder, 1937) have been shown to give rise to or be accompanied by a high blood potassium in the cat. The answer to the question of whether or not an inadequate oxygen supply plays a direct rôle in the present experiments must await more evidence, but the possibility that the mobilization of potassium is, in part, secondary to an action elsewhere is suggested by the fact that a rise in blood potassium also occurs in response to the injection of epinephrine (Bachromejew, 1932-33; D'Silva, 1934, 1936; Schwarz, 1935; Houssay, Marenzi, and Gerschman, 1936; Marenzi and Gerschman, 1936, 1937; Keys, 1938) and following central nervous stimulation (Cloetta, Fischer, and van der Loeff, 1934; Zwemer and Pike, 1938). The temporary effect of carbon dioxide excess may well be mediated through the nervous system and discharge of epinephrine.

It is also possible, as suggested above, that the labile blood potassium has a function in relation to the acid-base needs of the tissues, as, for example, in connection with the removal of acids formed in the interior of the cell during asphyxia or from penetration of carbon dioxide when given in excess. This phase of the problem is now being studied, particularly in relation to the effect of acid-base changes in the blood produced by the injection of

² Dennis and Moore (1938) found the potassium concentration in the coronary venous blood to be increased after ligation of the coronary arteries in cats.

acid, a procedure which decreases the serum potassium and largely prevents the asphyxial rise (Cattell, 1938). It is clear that much further information is needed before the significance of the potassium changes described in this paper can be appraised.

SUMMARY

The potassium content of the blood serum of cats has been studied in relation to its natural range of variability and the effect of various experimental procedures. The principal findings follow:

1. A wide range in potassium concentration (10 to 30 mg. per 100 cc.) occurred in apparently normal animals. Under "dial" anesthesia the potassium was only slightly more uniform but somewhat lower.

2. 4 to 5 minutes of complete asphyxia resulted in a marked, and when the initial values were low, several fold increase in the serum potassium, giving an average maximum concentration of about 38 mg. per 100 cc.

3. The serum potassium returned to the control values or below in from 2 to 3 minutes after the respiration was restored.

4. The breathing of a mixture of 10 per cent carbon dioxide and 90 per cent oxygen resulted in a temporary rise in blood potassium.

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THE DETERMINATION OF ASCORBIC ACID IN URINE WITH THE PHOTOELECTRIC COLORIMETER*

BY KENNETH A. EVELYN, HELGA TAIT MALLOY, AND
CHARLES ROSEN

*(From the Department of Medicine, McGill University Clinic, Royal Victoria
Hospital, Montreal, Canada)*

(Received for publication, September 8, 1938)

The main sources of error in the determination of ascorbic acid in biological fluids by titration with 2,6-dichlorophenol indophenol are due to difficulties in detecting the somewhat vague end-point, and to the presence of reducing substances other than ascorbic acid. The first of these errors may be eliminated by replacing visual titration by an objective photoelectric measurement of the amount of dye decolorized when a measured quantity of the solution reacts with an excess of dye (Rosen and Evelyn (1), Mindlin and Butler (2), Pijoan, Alexander, and Wilson (3)). In addition the use of the photoelectric method allows the measurement to be completed within 5 seconds, thus minimizing errors due to decolorization of the dye by interfering substances which react more slowly than ascorbic acid itself. In fluids such as plasma (2) and cerebrospinal fluid (3) the amount of interfering material is usually small, therefore accurate results can be obtained by making a single measurement at the end of 5 seconds. This is, however, far from being the case in urine, because non-ascorbic acid reducing substances often account for more than 90 per cent of the total indophenol-reducing capacity of normal urine. Attempts have been made (4-6) to remove the interfering substances by precipitation with mercuric acetate or barium acetate, but by these methods only a portion of such substances is removed.

It is the purpose of this paper to describe an alternative method of differentiating between ascorbic acid and other reducing sub-

* Aided by grants from The Banting Research Foundation, Toronto, Canada.

stances, which is based on differences in the rate at which the dye is decolorized. It has been shown that the reaction of ascorbic acid is almost instantaneous (7), while that of all other known interfering substances proceeds at a slower rate. This is further illustrated by the curves of Fig. 1 which show that the ascorbic

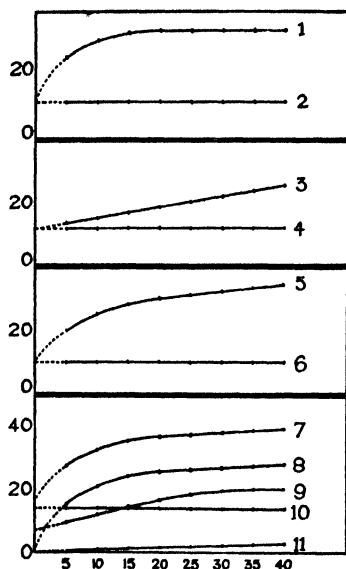


FIG. 1. Extrapolation of curves obtained from mixtures of reducing substances in order to obtain the true ascorbic acid content. Abscissa, time in seconds; ordinate, amount of dye decolorized in arbitrary units. Curve 1, ascorbic acid 0.0013 mg. per cc., thiosulfate 0.0056 mg. per cc.; Curve 2, ascorbic acid 0.0013 mg. per cc.; Curve 3, ascorbic acid 0.00143 mg. per cc., cysteine 0.050 mg. per cc.; Curve 4, ascorbic acid 0.00143 mg. per cc.; Curve 5, ascorbic acid 0.00116 mg. per cc., thiosulfate 0.0040 mg. per cc., and cysteine 0.0143 mg. per cc.; Curve 6, ascorbic acid 0.00116 mg. per cc.; Curves 7 to 11, representative samples of urine (Curve 10 was made with 0.1 cc. of urine of a patient who was undergoing an ascorbic acid saturation test).

acid reaction is complete within 5 seconds, while the presence of other reducing substances causes the reaction to continue beyond this time. In our method an excess of dye is mixed with a measured aliquot of urine, and the amount of dye decolorized is measured in the photoelectric colorimeter (8) at 5 second intervals until

the reaction is complete. The results are used to plot a reaction velocity curve from which the amount of decolorization due to ascorbic acid alone may be determined by extrapolation to zero time. This method is admittedly empirical and cannot therefore be expected to give more than approximate results, but the errors are far less serious than those of the ordinary visual titration which may often amount to several hundred per cent. Moreover, by combining the extrapolation procedure with a preliminary partial chemical purification we believe that measurements on urine can be made almost as accurately as on plasma.

Method

Freshly voided urine acidified with 5 per cent of glacial acetic acid by volume is used for all the determinations.

Reagents and Apparatus—

1. Aqueous solution of 2,6-dichlorophenol indophenol. No standardization of this solution is required other than to adjust its concentration so that 9 cc. of dye plus 1 cc. of 5 per cent acetic acid give a reading of about 30 on the photoelectric colorimeter with Filter 520.¹ The solution should be filtered, brought to approximately pH 7 by addition of a few drops of M/15 phosphate buffer, and stored in a brown bottle with a glass stopper.

2. A rapid delivery 9 cc. pipette is made by cutting off a suitable length from the lower end of an ordinary 10 cc. pipette, and recalibrating it to deliver 9 cc. The bore of the tip should be about 3 mm., so that the emptying time is about 1 second. A No. 6 rubber stopper is attached to the outside of the pipette at such a height that when it is inserted into a colorimeter tube the tip will be about 2 inches from the bottom of the tube (Fig. 2). The pipette must be rinsed after each determination to avoid contamination of the dye solution by traces of acid which may adhere to the tip.

Procedure

The initial optical density² of the dye solution is measured by adding 1 cc. of 5 per cent acetic acid to 9 cc. of dye in a colorimeter

¹ The colorimeter which we have used is manufactured by the Rubicon Company, 29 North Sixth Street, Philadelphia. Filter 520 is supplied with the instrument as standard equipment.

² The term optical density is used here to refer to the average optical

tube and reading immediately in the photoelectric colorimeter with Filter 520, after the galvanometer is adjusted to 100 with a tube containing water only. The corresponding optical density is recorded as L_1 . The galvanometer is then adjusted to 100 with a blank tube containing 9 cc. of water and 1 cc. of acidified urine; a sample tube containing 1 cc. of acidified urine is placed in the instrument, and 9 cc. of dye are run in from the special pipette which is held in place as shown in Fig. 2. The galvanometer is read at 5, 10, 20, and 30 seconds measured from the time at which addition of the dye is begun, the readings are plotted against time on graph paper, and the curve extrapolated by a smooth free-hand



FIG. 2. Rapid delivery 9 cc. pipette shown in place in the colorimeter tube just before delivery of the dye.

curve to intersect the axis of ordinates. The optical density corresponding to this extrapolated galvanometer reading is recorded as L_2 , and the concentration of ascorbic acid in mg. per 100 cc. of acidified urine is calculated from the equation

$$X = \frac{10.8 (L_1 - L_2)}{(1)}$$

density which is measured in the photoelectric colorimeter, as distinct from the true monochromatic optical density which is measured by the spectrophotometer. This approximate optical density is represented by the symbol L , and is similar to true optical density in that it obeys the relation $L = K \times C$ where K is a constant and C is the concentration of the colored substance in the solution.

where A cc. is the volume of acidified urine used in the test, and 10.8 is the numerical value of the calibration constant which has been determined by measurements on solutions of pure ascorbic acid.³

In measurements on urine it is very seldom necessary to use more than 1 cc. of urine, but the urine may have to be diluted before the test is made in order to limit the excursion of the galvanometer during the first 5 seconds to a maximum of 20 mm.⁴ If the ascorbic acid content of the sample solution is below 0.5 mg. per 100 cc., as for example in plasma filtrates, 1 cc. of a 10 times stronger dye solution should be placed in the colorimeter tube, and 9 cc. of the sample solution run in from the pipette. Equation 1 is of course accurate only as long as the final volume is 10 cc., and the 9:1 ratio is necessary to insure adequate mixing. In adapting the method to other fluids the solutions should be acidified or buffered so that the final pH of the dye mixture is about 3.

Results

Recovery of Ascorbic Acid—Table I illustrates the accuracy with which the true ascorbic acid content of mixtures containing varying amounts of interfering substances can be determined by the extrapolation method described above. The close agreement between the results obtained by independent observers shows that the somewhat empirical process of extrapolation can be carried out with a relatively small personal error. The complete failure of the titration method to differentiate between ascorbic acid and thiosulfate is well shown by the figures in the last column. It

³ The numerical value of this constant applies only to the particular type of photoelectric colorimeter used by us (Rubicon Company, 29 North Sixth Street, Philadelphia), but no difficulty would be experienced in adapting the method to any other type of instrument, other than the necessity for redetermining the value of the constant.

⁴ This precaution is necessary to allow for the lag in the galvanometer response due to inertia of the moving system. With the instrument used by us a movement of 20 mm. is the maximum which can be followed faithfully in 5 seconds. It is important that each new instrument used for this technique be checked by making a measurement on a solution of pure ascorbic acid of such a strength that a movement of 20 mm. is obtained. There must be no movement of the galvanometer spot or needle after the first 5 seconds.

TABLE I

Recovery of Ascorbic Acid from Mixtures Containing Other Reducing Substances

The figures in the two columns headed "Photoelectric" are the results obtained by two independent observers. In order to give the titrimetric method every advantage all titrations were carried out in the photoelectric colorimeter to an end-point in exactly 30 seconds.

Material in sample				Ascorbic acid determined		
Sodium thiosulfate	Cysteine hydrochloride	Urine	Ascorbic acid	Photoelectric		Titration
micrograms	micrograms	cc.	micrograms	micrograms	micrograms	micrograms
23			0	0.4	0	13.9
25			0	0.6	1.2	16.0
26			0	0.3	0.7	16.0
37			0	1.2	1.2	18.0
41			0	0.8	0.4	24.2
41			0	2.0	2.5	25.2
25			15.8	13.1	13.1	31.6
40			9.1	7.2	9.5	26.4
40			19.6	23.2	20.2	47.0
	143		0	0	0	2.4
	153		0	0	0	1.6
	153		0	0	0	2.0
	286		0	0.2	0	3.9
	143		10.8	10.7	9.3	16.8
	286		15.8	15.9	15.7	39.0
	306		14.5	13.7	13.1	14.5
40	143		10.8	7.2	11.3	36.5
24.8	153		12.5	14.3	13.7	31.0
24.8	153		11.0	7.2	9.5	28.6
24.8	153		8.5	11.9	10.5	27.2
24.8	153		14.5	16.7	14.6	21.4
		1.0	19.0	16.7	18.0	
		1.0	19.0	15.9	16.7	
		0.5	10.4	9.2	9.2	
		1.0	10.4	10.8	10.5	
		1.0	10.4	13.2	13.3	
		0.5	10.4	9.8	9.8	

should be mentioned at this point that, although we have used cysteine and thiosulfate as examples of typical interfering substances, we do not believe that they are necessarily the most important factors in human urine, because even when these two

substances are removed there still remains a variable amount of non-ascorbic acid reducing material.

Comparison of Titration and Photoelectric Methods in Urine—Table II shows the results obtained by measurements on fresh samples of urine from forty-five normal individuals whose diets were apparently adequate in ascorbic acid content. The figures

TABLE II

Comparison of Photoelectric and Titrimetric Methods of Determining Ascorbic Acid Concentration in Urine

The conditions of the experiments were the same as in the experiments reported in Table I. The results are expressed in arbitrary units.

Photoelectric		Titration	Photoelectric		Titration
85	85	114	4	8	104
40	40	106	22	25	100
2	3	30	35	33	94
19	19	51	38	35	120
30	29	85	32	38	114
28	28	61	10	9	85
170	170	202	9	9	64
65	65	109	31	40	97
38	38	73	98	97	178
50	50	74	76	74	157
100	100	141	125	135	271
13	13	33	43	40	89
140	140	172	63	62	120
50	50	113	90	89	211
23	23	75	90	111	229
130	130	160	75	75	170
100	100	167	20	16	58
32	32	79	0	10	148
23	23	60	0	40	315
2	0	25	85	100	297
170	170	179	60	50	179
			25	28	111

show that the ratio of titrimetric to photoelectric values varies from almost 1:1 to 10:1 and more. In view of the fact that a considerable percentage of normal individuals seems to excrete only traces of ascorbic acid in the urine, in spite of the apparent presence of relatively large amounts as determined by titration, it seems necessary to reexamine the significance of the urinary

output of ascorbic acid as an index of "vitamin C subnutrition." Our findings in this connection will be reported elsewhere.

Removal of Interfering Substances—In view of the magnitude of the errors caused by the presence of interfering substances in urine, and realizing that the extrapolation correction can only partially eliminate this type of error, we tried to combine the extrapolation procedure with the mercuric acetate and barium

TABLE III

Effect of Removal of Interfering Substances by Precipitation by Mercuric Acetate and by Solid Barium Acetate

All measurements were made by the photoelectric method. The figures in the columns headed "Ascorbic acid" were obtained by the extrapolation procedure described in this paper. The figures under the heading "Interfering substances" represent the ascorbic acid equivalent of the amount of dye decolorized during the first 30 seconds after the end of the true ascorbic acid reaction. The values are given in mg. per 100 cc.

Before precipitation		Solid barium acetate		Mercuric acetate	
Ascorbic acid	Interfering substances	Ascorbic acid	Interfering substances	Ascorbic acid	Interfering substances
0.3	1.5	0.2	0.7	0.5	1.4
0.3	1.3	0.2	0.7	0.4	0.8
0.4	0.8	0.3	0.5	0.3	0.4
0.5	0.3	0.4	0.2	0.5	0.1
0.8	0.7	0.7	0.2	0.6	0.4
1.0	0.8	0.9	0.4	1.1	0.3
1.1	1.6	1.0	0.3	0.9	0.3
1.2	2.8	1.4	1.2	1.2	1.7
2.2	0.7	1.9	0.5	2.3	0.6
2.9	2.2	3.2	1.7	2.8	0.9
4.2	0.5	3.7	0.3	4.4	0.4
7.0	0.3	6.6	0.3	7.0	0.3

acetate purifications of van Eekelen and his coworkers (4-6). The shape of the reaction velocity curves of the filtrates obtained by these procedures showed that they still contained a variable (though usually much smaller) amount of non-ascorbic acid reducing substances. Moreover, the mercuric acetate filtrates which had been reduced with hydrogen sulfide occasionally contained more of these interfering substances than the original

urine, no doubt due to the reduction by the hydrogen sulfide of substances present in the original urine (other than dehydroascorbic acid) in a harmless oxidized form. From Table III it may be concluded that the barium acetate precipitation is of definite value, since it always removes a portion of the interfering substances without causing any loss of ascorbic acid. There seems to be no advantage in using the mercuric acetate-hydrogen sulfide procedure, since in addition to the possibility of errors arising in the manner described above, it is tedious and may easily cause loss of ascorbic acid unless it is carried out very rapidly. Reduction with hydrogen sulfide must, however, be carried out on any solution which is known to contain appreciable amounts of dehydroascorbic acid.

In conclusion it may be mentioned that one of the most important applications of the method described in this paper is qualitative rather than quantitative. A study of the shape of the reaction velocity curve obtained from any solution affords a sensitive test of the ratio of interfering substances to true ascorbic acid, and may therefore be employed as a test of the efficiency of any method suggested for the removal of interfering substances. We recommend that this test be applied to all solutions, even those such as plasma filtrates in which, as a rule, the interfering substances are relatively unimportant.

SUMMARY

1. A method is described for the determination of ascorbic acid in urine and other biological fluids, in which titration has been replaced by an objective photoelectric measurement of the amount of dye decolorized when a measured quantity of urine reacts with an excess of dye.

2. This method does not require standardization of the dye solution, eliminates errors due to interfering colored substances, and allows the measurement to be completed within 5 seconds after addition of the dye. This greatly reduces errors due to non-ascorbic acid reducing substances, and a simple extrapolation procedure makes it possible to reduce this error still further.

3. As compared with the values obtained by the new method the titration values for normal urine are apparently often from 2

to more than 10 times too high, although the titrimetric method is quite accurate when the ascorbic acid content of the urine is very high.

4. The mercuric acetate purification of Emmerie and van Eekelen has not been found of value in connection with the photoelectric method of measurement, but preliminary precipitation with solid barium acetate is apparently both safe and desirable although it only removes a fraction of the interfering material.

5. With the new method 24 hour ascorbic acid excretions of less than 5 mg. are often found in normal healthy individuals on adequate well balanced diets.

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MICRODETERMINATION OF OXYHEMOGLOBIN, METHEMOGLOBIN, AND SULFHEMOGLOBIN IN A SINGLE SAMPLE OF BLOOD*

BY KENNETH A. EVELYN AND HELGA TAIT MALLOY

(From the Department of Medicine, McGill University Clinic, Royal Victoria Hospital, Montreal, Canada)

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The purpose of this paper is to present a simple, accurate photo-electric method for the determination of oxyhemoglobin (HbO_2), methemoglobin (MHb), and sulfhemoglobin (SHb) in a single sample of finger blood.

Methods

Methemoglobin—When sodium cyanide is added to a solution of MHb (Fig. 1), the characteristic absorption band at $635 \text{ m}\mu$ is almost completely abolished by the conversion of MHb into cyanmethemoglobin (MHbCN). The resulting change in optical density is directly proportional to the concentration of MHb. This change in optical density may be measured on the photoelectric colorimeter (1) with a color filter which transmits a narrow spectral band in the vicinity of $635 \text{ m}\mu$ (Fig. 1).

Sulfhemoglobin—The depth of the $620 \text{ m}\mu$ absorption band of SHb (Fig. 2) is unchanged by the addition of cyanide, hence the concentration of SHb in a solution containing HbO_2 , MHb, and SHb is proportional to the residual optical density of the solution after the MHb has been converted into MHbCN by addition of cyanide. This measurement can be made on the photoelectric colorimeter with a suitable filter (Curve 620 of Fig. 2) on the same solution used for the MHb determination. A correction must of course be made for the small, though not negligible, absorption of HbO_2 and MHbCN at $620 \text{ m}\mu$.

* Aided by grants from The Banting Research Foundation, Toronto, Canada.

Oxyhemoglobin—The concentration of HbO_2 is obtained by subtracting the values for MHb and SHb from the concentration of total hemoglobin determined by a modification of the method of Austin and Drabkin (3). In this method the various forms of hemoglobin are converted into MHbCN and the concentration of this pigment is determined by measuring the optical density of the solution in the photoelectric colorimeter, with a filter which

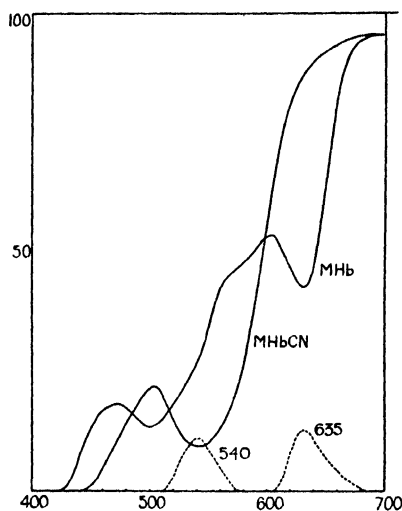


FIG. 1. Spectrophotometric curves of methemoglobin, cyanmethemoglobin, and Filters 635 and 540. These curves were made on solutions containing 0.1 gm. of hemoglobin per 100 cc. on the Hardy photoelectric spectrophotometer with a $10\text{ m}\mu$ slit; therefore they are presented by way of illustration of the principles of the method only, and not as accurate data on the spectrophotometric characteristics of these pigments. Abscissa, wavelength in $\text{m}\mu$; ordinate, percentage light transmission.

transmits a spectral band in the vicinity of the MHbCN absorption maximum at $540\text{ m}\mu$ (Fig. 1).

Reagents—

1. $\text{M}/15$ phosphate buffer of pH 6.6.
2. $\text{M}/60$ phosphate buffer of pH 6.6 prepared from the above by dilution as required.
3. 20 per cent aqueous potassium ferricyanide.
4. 10 per cent aqueous sodium cyanide.

5. A neutralized solution of sodium cyanide prepared within 1 hour of the time of use by mixing equal parts of 10 per cent sodium cyanide and 12 per cent acetic acid.

6. Concentrated ammonium hydroxide.

Reagents 3 to 6 should be kept in dropper bottles which deliver approximately 25 drops to the cc.

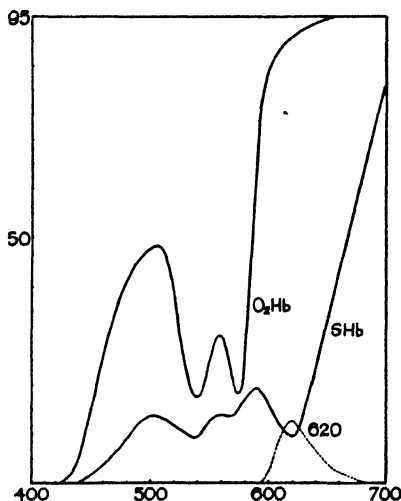


FIG. 2. Spectrophotometric curves of oxyhemoglobin (0.1 gm. per 100 cc.), sulfhemoglobin (0.15 gm. per 100 cc.), and Filter 620. The curve for SHb was drawn from the data of Drabkin and Austin (2). Abscissa, wavelength in $m\mu$; ordinate, percentage light transmission.

Procedure

0.1 cc. of fresh whole blood (finger blood, or venous blood to which not more than 2 mg. of potassium oxalate per cc. have been added) is delivered into 10 cc. of $M/60$ phosphate buffer of pH 6.6 in a colorimeter tube. The solution is allowed to stand for 5 minutes, and a reading is made with Filter 635,¹ after the galvanometer is first adjusted to 100 with a blank tube containing

¹ The photoelectric colorimeter and all accessories such as filters and absorption test-tubes can be obtained from the Rubicon Company, 29 North Sixth Street, Philadelphia.

water only. The optical density² is recorded as L_1 . 1 drop of the neutralized sodium cyanide is then added to the solution to convert any MHb into MHbCN, and after 2 minutes a second reading (L_2) is made with the same filter and blank tube. The difference ($L_1 - L_2$) is proportional to the concentration of MHb. The solution, which up to this time will have been very slightly turbid, is now cleared for the SHb determination by addition of 1 drop of concentrated ammonium hydroxide, and a reading (L_3) is made with Filter 620 and a blank tube containing water only. Finally 2 cc. of the solution are pipetted into a second colorimeter tube containing 8 cc. of M/15 phosphate buffer of pH 6.6 and 1 drop of 20 per cent potassium ferricyanide. The tube is allowed to stand for 2 minutes in order to allow all the HbO_2 to be converted into MHb; then 1 drop of 10 per cent sodium cyanide is added to convert the MHb into MHbCN for the total hemoglobin determination. At the end of 2 minutes a reading (L_4) is made with Filter 540 and a blank tube containing 10 cc. of water and 1 drop each of 20 per cent potassium ferricyanide and 10 per cent sodium cyanide. The concentrations of the various hemoglobin derivatives are calculated from the following equations in which T , M , and S represent gm. per 100 cc. of blood of total hemoglobin, MHb, and SHb respectively.³

$$T = \frac{100 \times L_4}{2.38} \quad (1)$$

$$M = \frac{100 (L_1 - L_2)}{2.77} \quad (2)$$

$$S = \frac{1000 \times L_3 - (8.5 \times M + 4.4 \times T)}{100} \quad (3)$$

² Throughout this paper the symbol L will be used to refer to the *approximate* optical density which is measured when one uses the photoelectric colorimeter, instead of a spectrophotometer which measures the true monochromatic optical density. With the filters used by us, however, the L value of a solution behaves just like the true optical density in that it is directly proportional to the concentration of the colored substance, and may therefore be used in the equation $L = K \times C$ where K is a constant and C is the concentration of the colored substance.

³ At the conclusion of the hemoglobin determination there remain 8 cc. of diluted blood which may conveniently be used for the determination of sulfanilamide by a simple photoelectric modification of the method of Marshall (4).

The value for T obtained from Equation 1 is not strictly accurate when the blood contains SHb, because it is not possible to convert SHb into MHbCN with the reagents used. Fortunately this error is not very serious, because the absorption (at 540 $m\mu$) of the compound formed from SHb by the action of ferricyanide is almost as great (78 per cent) as that of a corresponding amount of MHbCN. Since the error introduced in the total hemoglobin determination is directly proportional to the concentration of SHb, the true value of T may be calculated from the formula

$$\text{Corrected } T = T \text{ (calculated from Equation 1)} + 0.22 \times S \quad (4)$$

The concentration of oxyhemoglobin is obtained from the equation

$$\text{HbO}_2 = \text{corrected } T - (M + S) \quad (5)$$

The numerical values of the calibration constants which appear in the above equations apply only to the particular type of photoelectric colorimeter used by us, but the form of the equations will be the same for all photoelectric colorimeters, and no difficulty should be encountered in adapting the method to some other instrument.

Results

Data on the accuracy of the determination of the various hemoglobin derivatives are summarized in Table I. The measurements were made on artificial mixtures of known amounts of pure solutions of the individual pigments. Pure SHb was not available but solutions containing about 70 per cent SHb were prepared, and the concentration of SHb as determined by the method of Drabkin and Austin (2) was used in calculating the concentration of SHb in the final mixtures. From this data the following conclusions may be drawn.

Total Hemoglobin—When no SHb is present, the error of this determination does not exceed 0.2 gm. per 100 cc. When Equation 4 is employed to correct for the systematic error due to the presence of SHb, the error from this source can be kept below 0.4 gm. per 100 cc. even when the concentration of SHb is as high as 6 gm. per 100 cc. The highest concentration of this pigment which we have observed in four cases of sulfhemoglobinemia caused

by sulfanilamide is 1.1 gm., and in five cases of sulfhemoglobinemia due to other causes 2.5 gm. per 100 cc.

Methemoglobin—The error in this determination does not exceed 0.2 gm. per 100 cc. and is usually about 0.1 gm. The lower

TABLE I

Accuracy of Analyses of Mixtures Containing Oxyhemoglobin, Methemoglobin, and Sulfhemoglobin, with and without Addition of Other Non-Hemoglobin Pigments

All values are given in gm. per 100 cc. of blood.

Oxyhemoglobin		Methemoglobin		Sulfhemoglobin		Total hemoglobin	
Calculated	Measured	Calculated	Measured	Calculated	Measured	Calculated	Measured
9.8	9.7	0.2	0.3	0.0	0.1	10.0	10.1
7.6	7.4	2.4	2.5	0.0	0.0	10.0	9.9
5.8	5.6	4.2	4.2	0.0	0.1	10.0	9.9
0.5	0.5	9.5	9.5	0.0	0.0	10.0	10.0
8.2	8.3	1.2	1.4	0.6	0.5	10.0	10.2
8.0	7.9	0.3	0.3	1.7	1.6	10.0	9.8
6.5	6.2	1.5	1.5	2.0	2.1	10.0	9.8
4.4	4.6	2.1	2.0	3.5	3.7	10.0	10.3
2.2	1.8	1.8	1.6	6.0	6.2	10.0	9.6
9.8	9.6	0.2	0.2	0.0	0.1	10.0	9.9*
9.8	9.7	0.2	0.1	0.0	0.0	10.0	9.8†
9.8	10.0	0.2	0.1	0.0	0.1	10.0	10.2‡
4.4	4.3	2.1	2.2	3.5	3.7	10.0	10.2*
4.4	4.5	2.1	1.9	3.5	3.6	10.0	10.0†
4.4	4.6	2.1	2.1	3.5	3.7	10.0	10.4‡

* Evans' blue was added to blood in a concentration of 1.5 mg. per 100 cc. This corresponds to 5 times the maximum concentration used in the determination of blood volume by the method of Gibson and Evans (5). A patient who had received this amount of dye would be intensely cyanosed.

† To a sample of this blood was added the purple pigment obtained by exposing a solution containing 10 mg. of sulfanilamide per 100 cc. to the sun for 60 minutes. This pigment is either identical with or at any rate closely related to the pigment which occurs in the blood of patients receiving sulfanilamide, and the amount added in this experiment was sufficient to produce a much greater degree of cyanosis than is seen clinically.

‡ Phenolsulfonephthalein was added, 0.5 mg. per 100 cc. of blood.

limit of sensitivity is about 0.2 gm. per 100 cc., and this limit is partially due to the slow spontaneous formation of MHb which occurs when a 100-fold dilution of blood is allowed to stand.

Since the measurement is based on the difference of two optical densities, the accuracy is not affected by the presence in the solution of other pigments, even if they absorb light at $635\text{ m}\mu$, as long as their absorption is not altered by addition of cyanide.

Sulfhemoglobin—The sensitivity of the method is about 0.1 gm. per 100 cc. It is difficult to make an estimate of the absolute accuracy of the determinations, since the value of the calibration constant which appears in Equation 3 is obtained from the admittedly approximate data of Drabkin and Austin (2). It is probable, however, that the error in the concentration of SHb does not exceed 10 per cent of the value obtained by the above procedure. The reproducibility of the determination is almost as good as that of the total hemoglobin and MHb measurements, and the absolute accuracy can easily be improved at any future time if a method of preparing pure SHb should be discovered.

Effect of Extraneous Pigments—By the method described above any pigment which absorbs light at $620\text{ m}\mu$ (other than the hemoglobin derivatives themselves which are allowed for in Equation 3) will obviously be measured as sulfhemoglobin. A negative SHb test is always reliable, but a false positive test might conceivably be obtained on a blood which contained some other colored substance. This possibility is particularly important in the blood of patients receiving sulfanilamide, because this substance frequently gives rise *in vivo* to blue- and purple-colored derivatives which produce intense cyanosis. Fortunately, however, the interfering effect of such substances is almost entirely eliminated, since the blood is diluted 100-fold before the colorimetric measurements are made. Table I shows that the addition to blood of amounts of blue dyes several times greater than that required to produce alarming cyanosis has no appreciable effect on the accuracy of the determinations; therefore it seems fairly safe to conclude that the specificity of the method is adequate for use in human blood. If necessary, it might be possible to remove the interfering substance by using washed red blood cells instead of whole blood for the determination. It is recommended, however, that all bloods giving a positive SHb test be examined spectroscopically for the $620\text{ m}\mu$ band which is not affected by addition of cyanide and shifts its position when the blood is equilibrated with carbon monoxide. As an illustration of the satisfactory use of the method

in clinical investigation, it might be mentioned that in over 100 photoelectric examinations of blood from patients receiving sulfanilamide (in whom the indication for requisitioning the determination was obvious cyanosis) only four positive SHb tests were obtained (0.3 to 1.1 gm. per 100 cc.), and none of these was a false positive, since all four were confirmed spectroscopically. The incidence of methemoglobinemia was much higher, practically all the patients showing at least a trace of methemoglobin, but the amounts found were usually of the order of 0.5 to 1.5 gm. per 100 cc., and in only five cases over 2.5 gm. per 100 cc. The complete lack of correlation between the MHb concentration and the intensity of the cyanosis shows clearly that the usual cause of the cyanosis is the presence of colored derivatives of the sulfanilamide itself. Since this latter type of cyanosis is capable of masking that due to MHb completely, the determination of MHb is still of definite value, because methemoglobinemia *may* undoubtedly reach serious proportions in certain cases.

SUMMARY

1. A simple photoelectric method is described for the determination of oxyhemoglobin, methemoglobin, and sulfhemoglobin on a single 0.1 cc. sample of blood.

2. The determination of methemoglobin is subject to an error of not more than 0.2 gm. per 100 cc., and this also represents the smallest amount which can be detected with certainty.

3. As little as 0.10 gm. of sulfhemoglobin can be detected, but the absolute accuracy of the measurement is somewhat less than that of the methemoglobin determination.

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THE ISOLATION OF ESTRONE SULFATE FROM THE URINE OF PREGNANT MARES

By BENJAMIN SCHACHTER AND GUY FREDERIC MARRIAN

*(From the Department of Biochemistry, University of Toronto,
Toronto, Canada)*

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In an earlier communication (1), the present authors stated that they had obtained, by the fractionation of butanol extracts of the urine of pregnant mares, an amorphous water-soluble preparation containing about 40 per cent of chromogenic estrogen (calculated as estrone), which gave positive tests for inorganic sulfate after acid hydrolysis, and which did not contain glucuronic acid. It is the purpose of the present communication to describe in detail the methods employed for the preparation of such concentrates, and to report the isolation from one such preparation of a crystalline substance which has been satisfactorily identified as the potassium salt of estrone sulfate.

Throughout this work, the estrogen content of the urine specimens and of the various fractions obtained therefrom was determined by the colorimetric method of Cohen and Marrian (2), either directly, as was possible with some of the more purified concentrates of the conjugated estrogens, or after acid hydrolysis and extraction of the free estrogens. It is questionable whether the colorimetric method gives reliable values when applied to extracts, such as are obtained from mare urine, which contain a number of different estrogens, and therefore the figures reported for the "estrogen content (calculated as estrone)" of the various fractions must not be considered as being strictly quantitative. In spite of its limitations, however, the colorimetric method proved itself to be a satisfactory guide for the elaboration of purification procedures, and its use enabled the final object of this research to be achieved.

It was found that the free and conjugated chromogenic estro-

gens in pregnant mare urine could be readily extracted by means of butanol. Shaking such butanolic extracts with aqueous sodium hydroxide solutions was found to remove considerable quantities of unwanted impurities but little estrogen. This behavior of the conjugated estrogens in pregnant mare urine is in marked contrast to that of the estriol glucuronide in human pregnancy urine which is readily extracted from butanolic solutions by aqueous alkalis. Impurities such as benzoic acid, free estrogens, and other unconjugated ether-soluble compounds were then removed by thoroughly washing with ether the slightly acidified aqueous solution of the residue obtained by evaporation of the alkali-washed butanolic extract. The fraction containing the conjugated estrogens was recovered from the ether-washed aqueous solution by extraction with butanol, and evaporation of the butanolic extract after washing with sodium hydroxide and water. It was found to be advisable to conduct the removal of ether-soluble impurities as rapidly as possible and at a low temperature in order to minimize hydrolysis of the conjugated estrogens in the acid medium. A further degree of purification was achieved by dissolving the ether-insoluble, water-soluble material in chloroform with the aid of a small volume of ethanol, and extracting the solution with water. Considerable amounts of material remained in the chloroform phase, while the conjugated chromogenic estrogens were nearly quantitatively extracted.

Fractions obtained by the processes described above assayed from 15 to 65 per cent by weight of chromogenic estrogen (calculated as estrone). They were invariably amorphous, but varied in appearance greatly from batch to batch, being on some occasions white and powdery and on others deeply pigmented and gummy. The further concentration of the conjugated estrogens in such fractions proved to be a matter of very great difficulty. In some batches, precipitation with barium chloride from aqueous solution, followed by conversion of the precipitated barium salts into potassium salts, effected a considerable purification. In other batches this process appeared to be quite ineffective. From most batches it was found possible to remove a certain quantity of material with a very low chromogenic value by crystallization of the mixed potassium salts obtained directly from the sodium salts or by way of the barium salts. This procedure did not result in

any considerable concentration of the conjugated estrogens, but it was usually resorted to, since the material so removed was crystalline and appeared to be a nearly pure compound. The nature of this substance is now being studied in detail.

After removal of this compound, "crystallization" from ethanolic solution by the addition of ether was sometimes resorted to. In certain batches this procedure yielded white granular material of a high chromogenic content. In other batches, this procedure resulted in the formation of gels, the further treatment of which presented many difficulties.

From one batch of concentrate which had been subjected to all these procedures, a small amount of crystalline material was isolated, which melted over a range of 233–242° and which was shown to contain 74 per cent of chromogenic estrogen. It gave a negative Tollens test for glucuronic acid but after treatment with dilute hydrochloric acid it gave a positive test for inorganic sulfate. All the remaining material was used for a quantitative determination of organic sulfate. The result closely agreed with that required for the potassium salt of estrone sulfate. The ether-soluble material yielded in the hydrolysis for the sulfate determination was recovered and purified by sublimation in a high vacuum. It melted at 238° with the formation of a bright red color. It gave a positive Zimmermann (3) ketone test, and gave carbon and hydrogen analyses in close agreement with those required for estrone. The formation of the red color at the melting point suggested that the compound might be equilenin (Girard *et al.* (4)) or estrone contaminated with considerable quantities of that substance. However, the product gave a color in the Voss (5) test indistinguishable from that given by pure estrone, indicating that traces only of equilenin were present. There is no doubt therefore that the substance isolated was a slightly impure specimen of the potassium salt of estrone sulfate.

The procedures described in this paper provide a satisfactory method for obtaining partially purified concentrates of the water-soluble conjugated estrogens in mare urine. It must be admitted, however, that the methods employed for the isolation of estrone sulfate from such concentrates are still far from satisfactory, since they are not invariably effective and the yield is poor. The results submitted in this paper prove that the pregnant mare may con-

jugate estrone with sulfuric acid before excretion, but they do not exclude the possibility that conjugates of other types may also be excreted.

EXPERIMENTAL

Concentration of Conjugated Estrogens by Distribution between Butanol and Sodium Hydroxide Solution—3 volumes of 150 ml. each from the same sample of pregnant mare urine were treated respectively in the following manner. (a) The urine was acidified to pH 1.0 and allowed to stand at room temperature for 1 week to permit of hydrolysis of the conjugated estrogens. The hydrolyzed urine was then assayed for ether-soluble chromogenic estrogen by the usual Cohen-Marrian technique. A value corresponding to a total of 8.7 mg. of estrogen was obtained. (b) The urine was acidified to pH 3.0 and extracted four times with 50 ml. portions of butanol. The extract after being washed with water was evaporated to dryness, and the conjugated estrogens in the residue hydrolyzed by allowing it to stand in contact with 100 ml. of 0.1 N hydrochloric acid for 1 week. An assay of the ether-soluble estrogen in the mixture gave a value of 7.5 mg. It was therefore clear that the greater part of the total conjugated estrogen in the urine had been extracted by the butanol. (c) The urine was extracted with butanol as in (b). The extract after being washed with water was extracted six times with 50 ml. portions of N sodium hydroxide solution. The alkaline extract was then acidified to pH 1.0 and allowed to stand for 1 week in order to hydrolyze any conjugated estrogens present. An assay in the usual manner showed that the alkali had removed only 0.6 mg. of estrogen from the butanol.

Distribution of Alkali-Washed Butanol-Soluble Fraction between Water and Ether—16 liters of urine obtained from a mare at the 8th month of pregnancy were extracted three times with 5 liter portions of butanol. The combined extracts were washed three times with 1.5 liter portions of N sodium hydroxide, three times with 1 liter portions of water, and evaporated to dryness. The product weighed 18.4 gm. and gave a colorimetric assay corresponding to an estrogen content of 4.0 gm. This material was dissolved in 350 ml. of water and cooled in ice; the pH was adjusted to 3.0 with dilute hydrochloric acid, and the solution extracted

three times with 100 ml. portions of ether. The ethereal extract on evaporation yielded 2.3 gm. of solid which was found to contain 40 mg. of free chromogenic estrogen. This was discarded. The aqueous phase from the ether extraction was extracted four times with 120 ml. portions of butanol, and the extract so obtained, after being washed once with 50 ml. of *N* sodium hydroxide and three times with 50 ml. portions of water, was evaporated to dryness. The residue weighed 5.8 gm. and gave a colorimetric assay corresponding to 2.5 gm. of estrogen.

Distribution between Chloroform and Water—The final product obtained as described above was dissolved in 15 ml. of ethanol, and to the solution were added 200 ml. of chloroform. This mixture was then extracted five times with 35 ml. portions of water, and the combined aqueous extracts washed once with 50 ml. of chloroform. The chloroform-washed aqueous extract, on extraction with butanol and evaporation of the extract, yielded 3.3 gm. of material which gave a colorimetric assay corresponding to 2.15 gm. of estrogen.

Isolation and Identification of Potassium Salt of Estrone Sulfate—The material used was obtained from 9 liters of urine collected from a mare at the 8th month of pregnancy, by the methods of concentration described in the preceding sections. After solution in 30 ml. of water, saturated barium chloride solution was added until no more precipitate formed. The mixture was cooled in ice and the brownish, slightly gummy precipitate was filtered off and washed with ice-cold water.

The mixed barium salts so obtained were dissolved in 200 ml. of butanol, and after the solution was made slightly acid with dilute hydrochloric acid, it was washed once with 50 ml. of *N* potassium hydroxide and three times with water and evaporated to dryness. The mixed potassium salts obtained in this manner weighed 1.69 gm. and gave a colorimetric assay corresponding to 0.49 gm. of estrogen.

This material was then heated with 100 ml. of acetone and 2 ml. of water. A small amount of dark colored solid remained undissolved. This was filtered off and since it proved to be insoluble in both water and ethanol, it was discarded. The aqueous acetone solution was evaporated to dryness and the residue was extracted three times with 30 ml. portions of acetone to which a few drops

of water had been added. The insoluble material was again discarded. On slow evaporation of the aqueous acetone extract under a stream of nitrogen on the water bath, a small amount of white crystalline material separated out. After chilling, this was filtered off. A further quantity of similar crystalline material was obtained from the filtrate by further concentration. A total of 22 mg. of these crystals was removed in this way. Since the material gave a colorimetric assay corresponding to only 5 per cent of estrogen, it was set aside for future investigation.

After this crystalline substance was removed as completely as possible, the aqueous acetone solution was evaporated to dryness. The residue was dissolved in a small volume of ethanol and ether was added to the solution until a faint cloudiness was produced. This was redissolved by warming and the solution was stored overnight in the ice box. The white, gelatinous precipitate that separated was filtered off, washed with alcohol-ether, dried, and finally dissolved in the minimum quantity of butanol. After standing overnight in the ice box the small quantity of a white crystalline substance which separated out was filtered off, washed, and dried. This material weighed 6.5 mg. and melted over a range of 233–242° with some preliminary sintering. A colorimetric assay showed it to contain 74 per cent of estrogen (theoretical for K estrone sulfate is 69.6 per cent estrone). It gave a negative naphthoresorcinol test for glucuronic acid, and a positive test for sulfate after heating with dilute hydrochloric acid.

A quantitative determination of the content of $=\text{SO}_4$ sulfur was carried out in the following manner: 6.196 mg. in a small beaker were dissolved in 5 ml. of water. To the solution were added 2 ml. of *N* hydrochloric acid and 0.5 ml. of 10 per cent barium chloride solution, and the mixture was then evaporated to dryness on a boiling water bath. The residue was transferred to a platinum micro-Gooch crucible, washed with water, ethanol, and 1 per cent hydrochloric acid, and subsequently treated in the usual manner as recommended by Pregl.

6.196 mg. gave 3.645 mg. BaSO_4

$\text{C}_{18}\text{H}_{21}\text{O}_6\text{SK}$. Calculated, $=\text{SO}_4$ sulfur 8.26; found, $=\text{SO}_4$ sulfur 8.08

From the filtrate after the removal of the barium sulfate in the sulfur determination, the material liberated by the acid hydrolysis

was recovered by ether extraction after heavily diluting with water. The ethereal extract, after being washed with aqueous sodium carbonate and water, was evaporated to dryness. The small residue was purified as far as possible by sublimation at a pressure of 0.01 mm. and 130°. The product melted at 238° with preliminary sintering and with the formation of a bright red color. A colorimetric assay showed it to contain 97 per cent of estrogen (calculated as estrone). It gave a color in the Voss test indistinguishable from that given by the same weight of pure estrone. The Zimmermann ketone test was positive.

0.941 mg. gave 2.743 mg. CO₂, 0.701 mg. H₂O

C₁₈H₂₂O₂. Calculated, C 79.99, H 8.15; found, C 79.49, H 8.29

SUMMARY

1. A method for the preparation of concentrates of the water-soluble conjugated estrogens in the urine of pregnant mares is described. The products so obtained may have an apparent chromogenic estrogen content (calculated as estrone) varying from 15 to 65 per cent.

2. From one such concentrate has been isolated a small amount of a water-soluble crystalline substance that has been satisfactorily identified as the potassium salt of estrone sulfate contaminated with traces of equilenin.

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EFFECTIVENESS OF CHONDROITIN IN PREVENTING GIZZARD EROSION IN CHICKS*

By H. R. BIRD, J. J. OLESON, C. A. ELVEHJEM, AND
E. B. HART

*(From the Department of Biochemistry, College of Agriculture,
University of Wisconsin, Madison)*

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The existence of a dietary factor necessary for the normal development of the gizzard lining in young chicks has been reported previously from this laboratory, and it has been designated the anti-gizzard erosion factor (1-3). Assays of various plant and animal materials showed pig lung, liver, and kidney to be good sources, and oats, bran, and middlings to be superior to other grain products and much superior to corn. Green plant material such as dried grass and alfalfa leaf meal proved to be poor sources. Attempts to extract the active substance with water, ethyl alcohol, ether, and petroleum ether were uniformly unsuccessful, but dilute alkali removed the factor from lung tissue and acidification precipitated it along with the acid precipitable proteins. A reticulin preparation from lung tissue also proved to be potent, and this finding led to experiments with other connective tissues, among which cartilage proved most effective. Since chondroitin prepared from cartilage had been used in the treatment of stomach ulcer in humans by Crandall and Roberts (4), it appeared logical to try its effect on gizzard erosion. A preliminary report on its effectiveness has been presented previously (5). It is the purpose of this paper to describe these experiments in greater detail.

Almquist and Stokstad (6) and Almquist (7) have also described gizzard lesions in chicks fed a ration of polished rice, ether-extracted fish meal, ether-extracted brewers' yeast, salt, Wesson oil, and cod liver oil. They have reported prevention of erosion by the administration of hexane extracts of alfalfa leaf meal, kale,

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

wheat bran, and hemp-seed. They stated further that the active substance was present in the saponifiable fraction of hexane extract of alfalfa leaf meal. Numerous attempts on our part to repeat these results have served merely to confirm our original conclusion that alfalfa leaf meal is a very poor source and that the factor is not extractable by hexane from wheat bran or from any of the other materials which we have found to be good sources. These experiments have been performed not only with basal rations developed here, but also with the ration used by Almquist. Recently Almquist (8) has reported that bile, cholic acid, desoxycholic acid, sodium glycocholate, and sodium taurocholate possess gizzard factor potency.

EXPERIMENTAL

For the most part the details of the experimental procedure were the same as described previously, except that groups of five or six chicks were used, and all chicks were killed at 4 weeks of age. The practice of using small groups of chicks has been continued, since it permits a greater number of experiments; however, it necessitates the repetition of experiments yielding apparently significant results.

Table I gives the basal rations used in these experiments. 5 per cent of granite grit was added to these rations, in some cases during the last 2 weeks, and in some cases during the last 3 weeks of the experiment. All chicks were irradiated under a quartz mercury vapor lamp at a distance of 18 inches once a week, and given 10 mg. of either oleum percomorphum or haliver oil per chick per week. The oleum percomorphum contained 60,000 vitamin A units (U.S.P.) and 8500 vitamin D units (U.S.P.) per gm. The haliver oil contained not less than 50,000 vitamin A units (U.S.P. XI) and not less than 850 vitamin D units (U.S.P. XI) per gm. The composition of Salts I and the method of preparing the casein have been described previously (9). Two liver extracts were used, both being by-products of the preparation of anti-pernicious anemia factor. They were obtained from The Wilson Laboratories.¹ The brewers' yeast was obtained locally and dried.

¹ We are indebted to Dr. David Klein of The Wilson Laboratories, Chicago, for the liver extracts, cartilage, and chondroitin used in these experiments.

Most of the experiments reported here involved the feeding of Ration 465. Ration 466 was formulated in an attempt to secure better growth without reducing the severity of gizzard erosion. Previously published results were based on experiments with rations very similar to Ration 465, except that they contained 10 per cent of peanuts and no alfalfa leaf meal. Experience showed that peanuts were quite variable in their content of the anti-gizzard erosion factor, so the use of these rations was discontinued.

Table II gives a summary of the results obtained. Both the cartilage and the chondroitin were furnished by Dr. David Klein of The Wilson Laboratories. The chondroitin was a mixture of

TABLE I
Composition of Rations

Ingredients	Ration 465	Ration 466
Dextrinized corn-starch.....	64	57
Reprecipitated casein.....	18	18
Salts I.....	5	5
Brewers' yeast.....	1	1
Liver extract.....	2	2
Yellow corn.....		5
Wheat bran, heated at 120° for 24 hrs.....		5
Alfalfa leaf meal.....	10	5
Soy bean oil.....		2

chondroitin and chondroitinsulfuric acid containing some protein and mineral impurities. It strongly reduced Fehling's solution, probably indicating the presence of breakdown products of chondroitin. From this material the calcium chondroitin sulfate preparation was made by first treating with fullers' earth a solution of chondroitin in 0.2 N HCl, removing the fullers' earth by centrifuging, precipitating the chondroitin with 2 volumes of 95 per cent ethyl alcohol, filtering, redissolving in water, and subsequently precipitating with 1 volume of alcohol in the presence of acetic acid and excess of calcium acetate. The product was washed with successive portions of 50 per cent, 95 per cent, and absolute alcohol, and anhydrous ether. Later it was found advantageous to convert the material to the calcium salt before

TABLE II
Results of Assays for Anti-Gizzard Erosion Factor

Experiment No.	Supplement	Average weight, 3 wks.	Average weight, 4 wks.	Chicks with gizzard lesions		
				None or slight	Marked	Severe
Basal Ration 465						
1	None	82	103	2	8	4
	10% cartilage	123	158	13	0	0
2	None	80	99	0	4	2
	Hexane* extract \leq 10% cartilage	81	107	0	4	2
	“ residue \leq 10% “	114	147	6	0	0
3	None	82	105	2	4	2
	3% chondroitin (Wilson)	97	122	6	2	0
4	None	82	104	8	11	3
	5% chondroitin (Wilson)	86	112	16	3	0
5	None	74	100	0	5	1
	Hexane* extract \leq 5% chondroitin	71	94	1	2	3
	“ residue \leq 5% “	88	116	6	0	0
6	None	67	81	2	5	3
	3% chondroitinsulfuric acid (Meyer)	83	99	6	3	1
7	None	72	85	2	2	2
	3% Ca chondroitin sulfate preparation	84	110	6	0	0
	0.2 gm. per day Ca chondroitin sulfate preparation	87	107	4	2	0
	2% chondrosamine	59	74	2	3	1
8	None	80	99	0	4	2
	3% galactose	83	105	2	1	3
Basal Ration 466						
9	1% cod liver oil + 3% alcoholic extract of rice bran	74	96	0	5	2
	Same + 3% chondroitin (Wilson)	92	125	4	2	0
10	None	87	113	1	2	1
	3% chondroitin (Wilson)	101	128	5	1	0
	2% aldobionic acid preparation from gum arabic	102	131	5	0	0
	1% sodium glycocholate	80	106	5	1	0
	1% “ taurocholate	83	116	3	2	0
	0.5% cholic acid	101	140	5	0	0
	Ox bile \leq 2% solids	93	129	5	0	0

* Skelly-solve B.

treatment with fullers' earth. The resulting preparation gave no biuret test and only a very slight Fehling's test. The results of analysis were as follows: N 2.85, S 5.33, Ca 5.73, ash 24.43 per cent. Nitrogen was determined by the Gunning modification of the Kjeldahl method, sulfur by sodium peroxide fusion and subsequent conversion to BaSO_4 , and calcium by the method of the Association of Official Agricultural Chemists for calcium in a mineral feed. The calculated composition of chondroitinsulfuric acid completely converted to the calcium salt is: N 2.77, S 6.31, Ca 7.90, ash 26.8 per cent. The results indicate a mixture of the calcium salts of chondroitinsulfuric acid and chondroitin, relatively free of protein and mineral impurities.

The chondrosamine was prepared according to the method of Levene (10) from a barium salt prepared in a manner similar to that for the calcium salt described above.

The aldobionic acid preparation was made from gum arabic by the method of Heidelberger and Kendall (11), except that the acid was not crystallized. The product was reprecipitated twice and then fed as the syrup, which probably contained besides the glucuronogalactose some free glucuronic acid and some higher polymers.

Chondroitinsulfuric acid was prepared from cartilage by the method of Meyer and Smyth (12), except that the final precipitation in glacial acetic acid was omitted. Some difficulty was encountered in obtaining sufficient material for feeding.

DISCUSSION

The data in Table II afford ample support for the conclusion that chondroitin possesses gizzard factor potency. The difficulty of preparing very pure chondroitin makes it equally difficult to rule out the possibility of impurities accounting for the activity, but the following evidence may be cited as bearing on this possibility. In the first place, the protein-free calcium salt was more effective than the crude chondroitin from which it was prepared; secondly, extraction with hexane removed no potent material; and thirdly, two methods of preparation of chondroitin from cartilage yielded active material.

Aside from the possibility of impurities, there is also the ques-

tion as to whether a part of the chondroitin molecule would be effective. Chondrosamine may be ruled out on the basis of the data given in Table II, and the related sugar, galactose, also proved inactive. The glucuronic acid portion of the molecule has not been adequately tested. Prior to the experiments described here, glucuronic acid was fed at a level of 0.2 per cent of the ration, because it was known to be a constituent of various connective tissues. This relatively low level showed no activity, and higher levels have not been tried. The possibility that free glucuronic acid added to the ration may be destroyed before being assimilated by the chick requires consideration, since this is a rather labile compound. Of particular interest are the results of the feeding of the crude aldobionic acid fraction from gum arabic. This finding requires further investigation with a larger number of chicks, but the very definite effect obtained here would seem to indicate that glucuronic acid is involved in the gizzard erosion problem. Here also the possibility of impurities must be considered, but it does not seem very likely that the same impurities would be present in this preparation as in chondroitin. It is of interest to note that Crandall and coworkers (4, 13, 14) have pointed out the likelihood that glucuronic acid is the effective portion of the chondroitin molecule in alleviating gastric ulcer in humans and in promoting weight gains and improved nutrition in Eck fistula dogs. Manville *et al.* (15) have produced a glucuronic acid deficiency in rabbits by administration of menthol and have stated that, "Ulcerative and hemorrhagic defects were commonly found in the stomach, duodenum, gall bladder, and small and large intestine."

The apparent potency of the aldobionic acid preparation is also of interest in that it offers a possible explanation of the effectiveness of such plant materials as oats and bran in preventing gizzard erosion. The potency of the animal tissues which have been tried could be explained on the basis of their chondroitin content, but so far as we know, chondroitin has not been reported in plants.

The limited amount of data available on bile compounds is presented in confirmation of Almquist's recent report. Aside from the similarity in the lesions observed these data constitute the first evidence for the identity of the deficiencies observed by the California workers and by us. The data are not sufficiently

extensive to warrant conclusions as to the comparative effectiveness of these compounds. The use of bile in the treatment of peptic ulcer has been discussed recently by Emery and Schnitker (16). They found that it relieved the symptoms in a number of cases but concluded that their data were such as to exclude the use of desiccated ox bile as a specific cure for this disease. The possibility of a physiological relationship explaining the activity both of chondroitin and of bile would appear to be a very interesting one from the standpoint of further research. The effect of chondroitin in promoting weight gains in Eck fistula dogs observed by Crandall *et al.* may indicate such a relationship. Also it is well known that the liver plays a part in glucuronic acid metabolism, at least to the extent that detoxication by conjugation with glucuronic acid occurs there. As might be expected, the chicks receiving bile and bile compounds gave evidence of marked stimulation of bile secretion. The gallbladders were much enlarged and distended with bile. Further, it is common knowledge that bile contains mucin, which may explain on a chemical basis the protective activity of chondroitin and bile or bile salts.

The growth of the chicks in these experiments, as indicated in Table II, appears worthy of some comment. Almquist and Stokstad have made the statement that gizzard erosion does not affect growth. Our previous results led us to the opposite conclusion, although there was a possibility that growth differences were affected by complicating factors. Table II shows that, with the exception of the sodium glycocholate and taurocholate, every supplement preventing gizzard erosion also improved growth, although in some cases the differences were small and perhaps not significant. It seems likely that improvement in the basal ration to eliminate the possibility of other deficiencies would result in greater growth differences. A number of cases of the type of paralysis we have associated with vitamin B₄ deficiency were observed (17) and in nearly all cases these chicks were very much stunted in growth. As an example the group receiving Ration 466 + 3 per cent chondroitin may be cited. The average weight at 4 weeks given in Table II is 128 gm.; but if one chick weighing 55 gm. and exhibiting severe paralysis be eliminated, the average is raised to 143 gm. It is also of interest that

this was the one chick in the group showing marked gizzard erosion.

SUMMARY

Chondroitin preparations are effective in preventing gizzard erosion in chicks. Such animal tissues as have been found effective probably owe their potency to their chondroitin content.

The chondrosamine portion of the chondroitin molecule is not effective. The evidence that glucuronic acid is the active portion of the molecule is presented and discussed.

Confirmation is offered for Almquist's recent report on the activity of bile and bile compounds.

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THE DETERMINATION OF THE BUFFER ALKALI IN CUTANEOUS BLOOD*

By S. H. RUBIN

(From the Department of Chemistry, College of Medicine, and the Department of Biological Chemistry, Graduate School, New York University, New York)

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Greenwald and Lewman (1) described a method in which the protein and carbonic acid of blood are removed by picric acid, so that the alkali of the proteinates and bicarbonate is replaced by an equivalent of alkali picrate, which is measured. The picric acid precipitates the protein and frees the CO_2 , which is boiled off from the filtrate. The alkali picrate is measured as total picric acid minus free picric acid, and represents the buffer alkali of the proteinates and bicarbonate, called "titratable alkali" by Greenwald and Lewman.

As will be shown later, the "buffer alkali" measured by the picrate method includes nearly the total buffer alkali of plasma, but only about 80 per cent of that in the whole blood, the remainder being chiefly in the form of alkali salts of organic phosphates in the cells. The picric acid method nevertheless gives a useful measure of the alkali in the more important buffers of the blood.

Greenwald and Lewman titrated the excess picric acid with 0.01 or 0.005 N NaOH to end-points with several indicators—methyl red, phenol red, and thymolphthalein. The total picrate was estimated either gravimetrically as nitron picrate or by titration with titanous chloride. Sumner and Hubbard (2) subsequently used dinitrosalicylic in place of picric acid, since the former can be determined colorimetrically. The use of dinitrosalicylic acid has the disadvantage, however, that the titration of the acid to a

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fixed end-point (phenol red) is less exact than the corresponding titration for picric acid. We have, therefore, retained the latter as the precipitant of choice.

For the estimation of the small amounts of total picrate found in the "filtrate" from 0.1 ml. of blood, titration with dilute safranine solution in the presence of chloroform, as suggested by Castiglioni (3) was found to be accurate and rapid. Safranine reacts with picrates to form a reddish brown precipitate of safranine picrate which is moderately soluble in chloroform. Safranine and the alkali picrates are insoluble in chloroform. Bolliger (4) has similarly used methylene blue for the estimation of picrates. In parallel titrations with both dyes, we obtained sharper end-points with safranine.

Method

Solutions Required—

1. 0.1 per cent (about 0.003 M) safranine. 1.00 gm. of safranine is dissolved in 1 liter of water at room temperature and filtered. A few drops of chloroform are added as a preservative. When kept in a dark bottle, this solution retains its titer towards picric acid indefinitely. When exposed to diffuse daylight in a stoppered burette, it is stable for a month or so. The sample used in this work was labeled "Safranine O, Schultz 679" (National Aniline). This number refers apparently to the 1914 edition of "Farbstofftabellen;" in the edition of 1931, the number is 967. Rowe (5) classifies it as No. 841.

2. 1.2 per cent picric acid. Picric acid usually contains 10 per cent of added water. Dissolve 13.2 gm. of such picric acid in hot water in a liter volumetric flask, cool, and dilute to the mark.

3. 0.005 N NaOH, prepared daily by dilution of 0.1 N NaOH with CO₂-free water.

4. 0.03 per cent potassium oxalate, prepared daily by dilution from a stock 3 per cent solution adjusted to pH 7.4.

5. 0.01 per cent phenol red, prepared according to Clark (6).

6. Chloroform.

Technique

Receivers for the blood are prepared by pipetting 1.00 ml. of 0.03 per cent oxalate (Procedure A) or of water (Procedure B) into

3 ml. centrifuge or test-tubes. Semiautomatic capillary pipettes⁹ of the type described by Levy (7) have been found to be convenient at this and subsequent steps in the procedure where precise measurement of small volumes is required.

Collection of Blood. Procedure A—When only minimal amounts of blood are available, the samples are taken directly from the site of puncture into Folin microsugar pipettes (8) and transferred to receivers containing 0.03 per cent oxalate. With reasonable speed of operation, duplicate and often triplicate samples may be collected from a single incision. Application of moderate pressure to facilitate the flow of blood does not impair the accuracy of the results.

Procedure B—When 0.3 to 0.5 ml. (6 to 10 drops) of cutaneous blood can conveniently be collected, the sample is taken into a small test-tube containing 1 mg. of dry, neutral oxalate (prepared by evaporating 0.03 ml. of 3 per cent oxalate to dryness at 40–50°). 0.1 ml. samples of blood collected in this fashion are then pipetted into receivers containing exactly 1.00 ml. of water. For either Procedure A or B, the usual precautions regarding rinsing of the blood pipette should be observed.

Preparation of Centrifugate—Exactly 0.5 ml. of 1.2 per cent picric acid is added to the laked blood. The tube is stoppered with a clean rubber stopper or a cork which has been extracted with warm water, and shaken vigorously. Removal of the CO₂ formed by the action of picric acid on the blood bicarbonate is effected by alternate shaking and removal of the stopper for a few seconds. CO₂ determinations on bloods treated this way showed that negligible amounts remain after three or four shakings. After centrifugation, the supernatant fluid is taken into a 1 ml. pipette provided with a cotton plug. Adjustment to the mark is made after removal of the cotton and careful wiping of the pipette tip. The sample is then transferred to a test-tube (50 × 10 mm.) *provided with a pour-out*.

Titration of Free Picric Acid—After the addition of 0.01 ml. of 0.01 per cent phenol red, the contents of the tube are titrated with 0.005 N NaOH to the first appearance of a definite orange color (pH 7.3). The end-point is readily recognized by comparison with an untitrated tube held against a white background. Daylight or light from a daylight lamp is essential. The titration usu-

ally requires at least 0.2 ml. of the alkali, which may be measured with sufficient accuracy from a 1 or 2 ml. Koch microburette graduated in 0.01 ml. and capable of being read to 0.002 ml. A fine tip delivering 100 drops per ml. should be used.

Titration of Total Picrate—10 ml. of chloroform are poured into a glass-stoppered, French type separatory funnel having a total capacity of 50 ml. (15 ml. in the stem, 35 ml. in the bulb). The titrated blood filtrate is poured carefully into the separatory funnel and followed by four rinsings of the titration tube with 0.5 ml. portions of water. The transfer is not hazardous if the titration tube has a well rounded pour-out. The safranine solution is added from a 2 ml. microburette graduated in 0.01 ml., with occasional shaking of the separatory funnel to permit solution of the precipitated safranine picrate in the chloroform. Near the end-point, frequent renewal of the chloroform is necessary to avoid a premature end-point owing to the appreciable solubility of safranine picrate in water (0.0048 gm. per cent at 18° (3)). The end-point is taken as the first appearance in the aqueous layer of a pink color which is *unextractable* by fresh chloroform. Under these conditions, the end-point can be duplicated with a deviation of less than 1 per cent in repeated titrations of the same material. The titration usually requires 1 to 2 ml. of 0.1 per cent safranine. About 35 to 50 ml. of chloroform are used in each titration. This may be collected in a large bottle over CaCl_2 and distilled when a sufficient quantity has accumulated.

Standardization of Safranine—For the standardization of the safranine, a dilute picric acid standard, about 0.003 M, is required. This may be prepared conveniently by dilution from the 1.2 per cent (about 0.05 M) picric acid, after standardization of the latter by titration with standard alkali to a *phenol red* end-point. Towards methyl red, picric acid behaves strictly as a monobasic acid, the calculated value being obtained on titration with NaOH. Titration to phenol red, however, requires 1.020 milliequivalents of base per mm of acid. No correction in the safranine titer is necessary if the dilute picric acid standard is prepared as described.

Calculation— $b = 16,000 ((ac) - (de))$ where b = buffer alkali, in milliequivalents per liter of blood; a = ml. of safranine; c = normality of safranine; d = ml. of alkali; e = normality of NaOH (usually 0.005 N).

EXPERIMENTAL

Greenwald and Lewman used a dilution of 1:10 throughout their work and showed that at this dilution the buffer alkali is not affected by large variations in the amounts of oxalate or picric acid used, nor by alterations *in vitro* of the CO₂ content of the blood. In the course of the present study, it was found that with increasing dilution there occurs a progressive increase in the observed value of the buffer alkali when the ratio of volumes of picric acid to volumes of blood is kept low. Thus, 6 volumes of 1 per cent picric acid or 5 volumes of saturated picric acid will suffice

TABLE I

Effect of Dilution on Apparent Concentration of Buffer Alkali and Other Constituents of Normal Human Blood (Venous)

The results are reported in milliequivalents per liter, except for acid-soluble P which is measured in mm per liter.

Constituent determined	Material	Original blood	Dilution of filtrate			
			1:7	1:10	1:25	1:40
Buffer alkali.....	Blood		36.9	37.5	40.6	42.4
“ “	Plasma, venous		43.8	44.0		45.1
Total base.....	Blood	132		136	134	133
Chloride.....	“	80.8		83.3		81.2
Non-protein N.....	“			26.4		26.0
Amino N.....	“		7.0	7.2	6.9	7.0
Peptide N.....	“		2.9	3.0	2.9	2.8
Acid-soluble P.....	“			7.2		6.0

In these experiments, a constant ratio of 6 volumes of 1 per cent picric acid to 1 volume of whole blood was maintained. The effects of an increase in this ratio at the greater dilutions on the values of the buffer alkali and acid-soluble phosphate are discussed in the text.

for the complete precipitation of the proteins and lipids in 1 volume of normal human blood at any dilution up to about 1:40, but the apparent value of the buffer alkali is about 5 milliequivalents higher at the latter dilution than at 1:10 (Table I). Differences of this magnitude were observed in human blood (defibrinated, oxalated, or heparinized) as well as in defibrinated pig and beef blood. Values derived from analyses of the filtrates obtained at different dilutions are given in Table I.

The data show differences of about 3 per cent in total base, chloride, and non-protein nitrogen between 1:10 and 1:40, but these differences can undoubtedly be ascribed to a "protein volume displacement" effect; *i.e.*, the precipitated proteins occupy a definite volume which actually diminishes the volume of the filtrate and so increases the concentration of solutes, notably at the lower dilution. Increasing the dilution from 1:10 to 1:40 decreases the apparent cation concentration (total base) found by 3 milliequivalents per liter of whole blood while the anions, chloride and acid-soluble P, are decreased by 2.1 and 1.2 mM, respectively. If the acid-soluble P is estimated to represent 1.9 equivalents of acid per mole of P (Table IV) the anion decrease in milliequivalents is estimated as $2.1 + 2.3 = 4.4$, or 1.4 milliequivalents more than the total base decrease. This decrease in Cl plus organic P would therefore suffice to cause an increase of 1.4 milliequivalents in the alkali picrate of the whole blood filtrate. The observed increase in alkali picrate caused by the high dilution was, however, greater; *viz.*, $42.4 - 37.5 = 4.9$. There must be factors involved other than those considered in Table I, but the nature of these is not at present apparent. Electrometric (glass or quinhydrone electrode) titrations showed that neither the ionic strength nor the initial pH of the filtrate is significant in this respect.

When 8, instead of 6, volumes of 1 per cent picric acid are used in preparing the 1:40 filtrate, the difference (1.2 mM) in acid-soluble P is found to be completely abolished, and the difference in buffer alkali reduced from 5 to about 3 milliequivalents.

The following methods were used to obtain the data given in Table I.

Total base was determined by the method described by Peters and Van Slyke (9), with the difference that the benzidine sulfate was weighed on a microbalance.

Chloride was determined gravimetrically as AgCl^1 and volumetrically by the procedure of Sunderman and Williams (11).

¹ Greenwald and Gross (10) found that the silver chloride precipitated from picric acid filtrates of whole blood was contaminated with yellow purine-silver picrate, containing adenine and hypoxanthine. The purine-silver picrate was soluble in concentrated HNO_3 and changed to an insoluble form in NH_3 . The AgCl precipitates observed in the present work were free from this contaminant since (1) they were white, (2) they

Acid-soluble P was determined by the method of Fiske and Subbarow (12); a yellow filter (Jena GG11) was used in the colorimeter.

Non-Protein Nitrogen—Aliquots of the blood filtrates were concentrated to a suitable volume and then acidified with H_2SO_4 . After removal of the picric acid with benzene in a continuous extractor, the water-clear extract was submitted to a micro-Kjeldahl determination. Since the estimation of non-protein nitrogen was indirect, *amino* and *peptide nitrogen* were also determined; the procedure of Hiller and Van Slyke (13) was followed closely in urea-free filtrates.

Comparison of Direct and Indirect Estimates of the Buffer Alkali

As a check on the accuracy of the method of Greenwald and Lewman, the buffer alkali of the arterial blood and plasma of five normal young men was estimated both by this method and by summation of the base bound by the individual buffers. Available data permit a more accurate estimate of the latter in arterial than in venous blood.

After anesthetization of the wrist with 1 per cent novocaine, 40 ml. of blood were drawn, with anerobic precautions, into a syringe containing 0.1 ml. of a 4 per cent solution of colorless, "double strength" heparin. The blood was transferred over mercury to a 50 ml. sampling bulb and mixed. For separation of the plasma, 15 ml. were centrifuged under paraffin. The following estimations were made in duplicate or triplicate on the whole blood.³

lost no weight when washed with HNO_3 , (3) they were completely soluble in NH_3 , (4) the volumetric determinations agreed with the gravimetric within 1.5 per cent. The source and age of the bloods used in the experiments of Greenwald and Gross were not specified. It is possible that in bloods which are permitted to stand, the hydrolysis and deamination of adenylypyrophosphate liberates sufficient adenine and hypoxanthine to account for the observations of these authors. It should further be noted that in order to obtain correct results by the gravimetric method, it is essential that the silver chloride be washed with ether, since a small particle of undigested fat invariably remains after the usual treatment with hot nitric acid. This precaution was observed, though not noted, by Greenwald and Gross (personal communication from Dr. Greenwald).

³ The subscripts *b*, *s*, and *c* refer to whole blood, plasma, and cells, respectively. The parentheses denote concentrations per liter of blood, plasma, or cells.

$(O_2)_b$ and $(CO_2)_b$ —The procedure of Van Slyke and Neill (9) was followed on the manometric apparatus.

O_2 Capacity—About 4 ml. of blood were equilibrated with air in a rotating device for 25 minutes. 1 ml. samples were analyzed in the manometric apparatus. Dissolved oxygen was estimated from the solubility factors of Sendroy, Dillon, and Van Slyke (14).

Water $(H_2O)_b$ —2 ml. samples were weighed into Pyrex dishes containing sand and dried to constant weight at 100–105°.

Buffer alkali (b_b) , by the macrogravimetric method of Greenwald and Lewman at a 1:10 dilution. The free picric acid was titrated in a quinhydrone electrode to pH 7.30.

Cell volume, V_c , in the Daland hematocrit tube, with the precautions given by Eisenman, Mackenzie, and Peters (15).

The following determinations were made on the plasma.

pH_s, by the bicolor method of Hastings and Sendroy (16). The standard tubes were calibrated against Sørensen phosphate standards which had been checked with the hydrogen electrode.

Proteins (P_s) —0.5 ml. samples were submitted to a semimicro-Kjeldahl analysis. Non-protein nitrogen was taken as 25 mg. per 100 ml.

$(CO_2)_s$ —1 ml. samples were analyzed in the Van Slyke apparatus.

Buffer alkali, b_s , as above for b_b .

From the above, the following were calculated.

$(H_2O)_s$, from the equation of Eisenman, Mackenzie, and Peters (15), $W_s = 98.5 - 0.745 P_s$.

$(H_2O)_c$, from $(H_2O)_b$, $(H_2O)_s$, and V_c .

The distribution of H_2CO_3 and $BHCO_3$ between cells and plasma was determined with the aid of the constants and equations given by Dill, Daly, and Forbes (17) and Dill, Edwards, and Consolazio (18).

$$(H_2CO_3)_c = \frac{(CO_2)_c}{10^{pH - pK} + 1}$$

$$(BHCO_3)_c = (CO_2)_c - (H_2CO_3)_c$$

$$pCO_2 = \frac{(H_2CO_3)_s}{0.0334(H_2O)_s}$$

$$(H_2CO_3)_b = (0.0334 (H_2O)_s V_s + 0.0362 (H_2O)_c V_c) pCO_2$$

$$(BHCO_3)_c = \frac{(CO_2)_b - (H_2CO_3)_b - (BHCO_3)_s V_s}{V_c}$$

$$pH_c = pK'_c + \log \frac{(BHCO_3)_c}{(H_2CO_3)_c}$$

pK' was taken as 6.04 (17), pK' as 6.11 (17).

BHb , the base bound by hemoglobin and other non-dialyzable anions, was estimated from Table VII of Adair (19). BP_c , the base bound by hemoglobin and other non-carbonate buffers, dialyzable and non-dialyzable, in the cells, was estimated by the empirical equations developed by Dill, Edwards, and Consolazio (18). BP_p , the base bound by plasma protein, was calculated by the equation of Van Slyke, Hastings, Hiller, and Sendroy (20).

The data shown in Table II are in good agreement with other recent studies (18, 21) on the composition of normal human arterial blood. The values for $(H_2O)_c$ are somewhat higher and those for $(H_2O)_p$ somewhat lower than the data of Dill, Edwards, and Consolazio (18); this may be due to differences in methods.

The comparative values of the buffer alkali as well as an estimate of the total buffer alkali of whole blood are given in Table III. For both whole blood and plasma, the values obtained by the alkali picrate method are somewhat lower than those calculated from the titration curves of carbonic acid and the proteins, the average differences being 2.7 and 1.5 milliequivalents per liter of blood and plasma, respectively. These average figures are of the same magnitude as the unexplained fraction of the difference in buffer alkali due to dilution, and suggest that values of the buffer alkali determined at about 1:40 would be more nearly correct. On the other hand, it must be noted, in so far as the whole blood figures are concerned, that the estimates of $BHbO_2$, the base bound by oxyhemoglobin, are probably too high. The determinations of Adair (19) were made of the entire non-dialyzable constituents of the cells, which include, besides hemoglobin, other constituents which bind base. Corrections for the latter would reduce $BHbO_2$ and bring the calculated values of the buffer alkali (Table III) closer to those found by the picrate method at a 1:10 dilution.

Organic Cell Phosphates As Carriers of the Whole Blood Buffer Alkali in Excess of That Measured by the Picrate Method

The total buffer of whole blood includes, besides the buffer alkali measured by the picrate method, the base bound by the non-hemoglobin buffers of the erythrocytes. These consist largely of the acid-soluble organic phosphate compounds, diphospho-1-glyceric acid (22), adenosinetriphosphate (23), and one or more hexosephosphates (24, 25).

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Table IV contains two estimates of the base bound by these phosphate esters. The first estimate is based upon the data of Warweg and Stearns (26) who determined the phosphate components by a system of acid and enzyme hydrolysis. It has been noted by Bakwin, Bodansky, and Turner (27) and by Kerr and Antaki (28) that Warweg and Stearns overlooked the adenylic

TABLE III

Comparison of Titrated and Calculated Buffer Alkali in Milliequivalents per Liter of Blood or Plasma

Dilution of blood, 1:10.

Subject	Whole blood					Plasma		
	Bicarbonate and proteinate alkali			Total buffer alkali		Bicarbonate and proteinate alkali		
	Titred by picrate method (1)	Calculated* (2)	Difference, (3)-(1) (3)	Calculated† (4)	Difference, (4)-(1) (5)	Titred (6)	Calculated‡ (7)	Difference, (7)-(6) (8)
A. D.	38.7	40.7	2.0	48.0	9.3	41.1	42.7	1.6
S. R.	40.2	43.2	3.0	51.1	10.9	42.4	44.5	2.1
M. M.	38.1	40.2	2.1	47.8	9.7	41.2	42.6	1.4
W. C.	36.3	38.6	2.3	46.1	9.8	40.2	41.1	0.9
N. G.	36.9	41.1	4.2	48.6	11.7	41.0	42.7	1.7
Mean	38.0	40.7	2.7	48.3	10.3	41.2	42.7	1.5

* Calculated as the sum of alkali in whole blood bicarbonate, plasma proteinate, and non-dialyzable cell anions, chiefly hemoglobin. BHb = alkali bound to Hb and other non-dialyzable anions. $b_b = (HCO_3)_b + V_b(BP)_b + (BHb)_b$ (Table II and text).

† Calculated as the sum of alkali in whole blood bicarbonate, plasma proteinate, and total cell buffers; i.e., $BP_c = BHb +$ alkali in cell organic phosphates and other non-carbonate buffers of the cells, dialyzable and non-dialyzable. $B_b = (HCO_3)_b + V_b(BP)_b + (BP_c)_b$ (Table II and text).

‡ Calculated as the sum of alkali in plasma bicarbonate and proteinate. $b_p = (HCO_3)_p + (BP)_p$ (Table II and text).

acid phosphorus of the adenosinetriphosphate fraction and therefore underestimated the concentration of the latter. The corrected value appears in Table IV. The second estimate is based upon the data of Rapoport (29) and Guest and Rapoport (30) who determined glyceric acid by the method of Rapoport (29) and calculated phosphoglycerate therefrom. The remaining phos-

phorus is considered to be present largely as adenosinetriphosphate. This fraction probably includes some phosphorus belonging to the triphosphopyridine nucleotide studied by Warburg, Christian, and Griese (31), but the concentration of this compound is not sufficient to affect appreciably the values cited in Table IV.

The values of b' , the milliequivalents of base bound per mm of substance at pH 7.2, were taken from the following: for phosphoglycerate, from the titration shown in Fig. 1 and discussed below; for adenylypyrophosphate, from Lohmann (32); for hexosephosphate, from Meyerhof and Suranyi (33). Although the exact identity of the hexosephosphate in blood is unknown, the amounts of base bound by this component are relatively so small

TABLE IV
Estimates of Base Bound by Organic Phosphate in Red Cells

	b' at pH 7.2	Warweg and Stearns (26)			Guest and Rapoport (30)		
		P	Phos- phate ester	Base bound	P	Phos- phate ester	Base bound
	<i>m.eq.</i> <i>per mM</i>	<i>mg. per</i> <i>cent</i>	<i>mM per</i> <i>l. blood</i>	<i>m.eq.</i> <i>per l.</i> <i>blood</i>	<i>mg. per</i> <i>cent</i>	<i>mM per</i> <i>l. blood</i>	<i>m.eq.</i> <i>per l.</i> <i>blood</i>
Diphosphoglycerate	3.82	14.4	2.32	8.9	10.6	1.71	6.5
Adenylypyrophosphate . . .	3.80	5.7*	0.61*	2.3	9.5	1.02	3.9
Hexosephosphate	3.8	1.1*	0.2*	0.8	1.1	0.2	0.8
Total ester P		21.2		12.0	21.2		11.2

* Corrected; see text.

that little error is introduced by the use of the titration curve of a representative compound. The total base bound by all three substances amounts to 12.0 milliequivalents per liter of blood for Warweg and Stearn's distribution, 11.2 milliequivalents for Guest and Rapoport's. These figures agree favorably with the value 10.3 (range 9.3 to 11.7) given in Table III, Column 5, as the mean difference between the total buffer alkali (calculated as described above) and the buffer alkali of the proteins and bicarbonate determined by the picrate method. From this agreement, it may be inferred that the method of Greenwald and Lewman yields essentially correct values for whole blood at a dilution of 1:10. It is recognized that the calculations shown in Table IV are preliminary

in nature and would be vitiated if it should be shown subsequently that some of the base-binding groups assumed to be free occur in an esterified form in the blood. The stroma protein described by Jorpes (34) is isoelectric at pH 5.2, but it is present in too low concentration to bind appreciable amounts of base. The lipids, lecithin and cephalin, are zwitter ionic and do not bind base at physiological reactions (35-37).

Titration of Diphosphoglyceric Acid

The tribarium salt of diphosphoglyceric acid was prepared from fresh horse blood³ by the method of Greenwald (22). Anal-

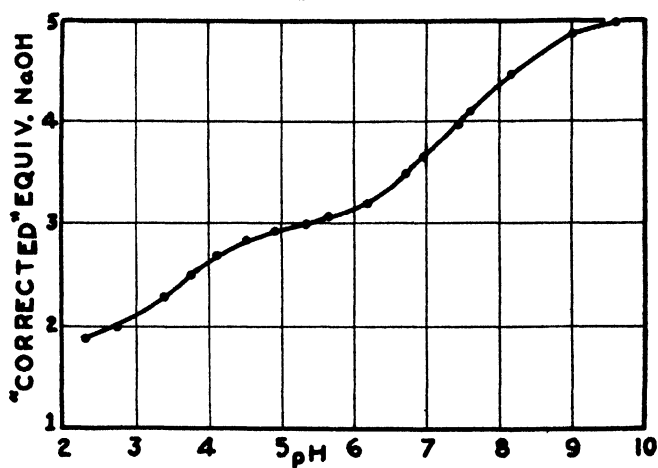


FIG. 1. Titration of diphospho-1-glyceric acid at 25°

ysis of the preparation gave Ba 43.4 per cent (as BaSO_4), P 13.03 per cent (as strychnine phosphomolybdate); calculated for $\text{Ba}_3(\text{P}_2\text{C}_3\text{H}_5\text{O}_{10})_2 \cdot \text{H}_2\text{O}$, Ba 43.1 per cent, P 12.97 per cent. The material contained 0.1 per cent nitrogen but gave a negative test for pentose with Bial's reagent. The free acid was prepared by treating weighed amounts of the tribarium salt with an equivalent amount of 0.993 N H_2SO_4 and filtering off the BaSO_4 formed. The filtrate was free from inorganic phosphate and sulfate. For

³ The Lederle Laboratories, Inc., at Pearl River, New York, kindly supplied the blood and facilities for deproteinization.

titration, the solution was diluted to about 0.0025 M and made 0.1 M with respect to KCl. 20 ml. samples were titrated with CO_2 -free 0.1080 N NaOH in a bubbling hydrogen electrode. Two different preparations from the tribarium salt gave titrations that agreed within 0.04 pH unit in the acid range, within 0.07 pH in the alkaline range. The titration curve is shown in Fig. 1. The "corrected" equivalents of base, b' , were calculated with the formula of Simms and Levene (38). The ionic strength varied from 0.11 at b' 3.0 to 0.12 at b' 5.0, which is within the range 0.10 to 0.12 given by Stadie and Hawes (39) as the ionic strength per liter of red cells. By means of the formulas of Meyerhof and Lohmann (40), pK'_4 was estimated to be 6.78 ± 0.04 , pK'_5 8.14 ± 0.05 . The data are not sufficiently accurate for the estimation of the more acid groups.

As a check on the electrometric procedure, several titrations were made of 0.01 M KH_2PO_4 (Sørensen salt) made up in 0.1 M KCl. The mean value of pK'_2 was found to be 6.80 ± 0.01 , which is in good agreement with the figure 6.82 given by Cohn (41).

After the work reported in this section had been completed, it was found that Kiessling (42) had titrated a preparation of diphosphoglyceric acid made from pig blood. Although the two titration curves are in fair agreement over the alkaline range, Kiessling's estimates of pK'_4 (7.46) and pK'_5 (7.99) are significantly different from those given above (6.78 and 8.14, respectively). Since the Meyerhof-Lohmann formulas were used in both instances, we are at a loss to reconcile the difference. Our own estimates from Fig. 2 of Kiessling's paper indicate values in agreement with our own.

Effect of Laking Blood

Most of the determinations of the buffer alkali considered in the previous sections were made by the macrogravimetric method. This method was also used to obtain the data of Table V, wherein it is shown that laked and unlaked samples of arterial blood yield the same values, and that allowing laked specimens to stand up to 30 minutes before adding picric acid does not affect the results appreciably. In collecting numerous samples for analysis by the micromethod, it often happens that the addition of picric acid is delayed for some time.

Precision of Micromethod

Sulfuric and lactic acids and sodium carbonate added to whole blood *in vitro* were recovered quantitatively (Table VI).

TABLE V

Effect of Standing on Buffer Alkali of Laked Human Arterial Blood

The results are expressed in milliequivalents per liter of blood.

Subject			Time laked, min.				
			1	10	15	20	30
D.	Unlaked	38.7					
	Laked		38.5	38.5	38.3		38.1
G.	Unlaked	36.9					
	Laked		37.4	37.0		37.2	36.9

TABLE VI

Effect of Acid and Alkali Added in Vitro on Buffer Alkali of Blood

The results are expressed in milliequivalents per liter of blood.

Blood	Buffer alkali	
	Observed	Calculated
Beef (fresh).....	38.0	
“ + 1.00 ml. 0.86 N lactic acid.....	29.9	29.4
“ + 2.00 “ 0.86 “ “ “.....	21.5	20.8
“ + 1.00 “ 0.484 N Na ₂ CO ₃	42.4	42.8
“ + 2.00 “ 0.484 “ “.....	48.0	47.7
Sheep (fresh).....	43.4	
“ + 1.00 ml. 0.484 N Na ₂ CO ₃	48.5	48.2
“ + 2.00 “ 0.484 “ “.....	53.0	53.1
“ + 1.00 “ 1.132 “ H ₂ SO ₄	32.5	32.1
“ + 2.00 “ 1.132 “ “.....	21.2	20.8

The indicated quantities of acid or alkali were added to 100 ml. portions of blood, samples of which were then precipitated with picric acid. The results, which are the means of quadruplicate determinations (at a dilution of 1:16) show complete recovery of added acid and alkali.

Comparative micro- and macroanalyses were made on fingertip and venous specimens,⁴ respectively, taken from thirty-nine

⁴ I am indebted to Professor Ralli of the Department of Medicine for permission to draw these bloods as well as for the rats used to obtain the data of Table VII.

individuals. Of these, thirty-one were normal young men, and eight were patients with a plasma CO_2 -combining power below normal. The average difference between the individual means was ± 0.5 milliequivalent, with a range of $+1.0$ (i.e., micro greater than macro) to -0.9 , with about the same variation in the normal and abnormal groups. The difference was positive in seventeen instances, negative in twenty-two. With signs taken into account, the mean difference was 0.1 milliequivalent or virtually zero, and hence the differences are probably not significant. This is borne out by calculation of t according to Fisher (43); t is equal to 0.79. For a probability of 0.01 (one chance in 100 that random sampling is responsible for the difference), the value of t

TABLE VII

Normal Values of Buffer Alkali in Whole Blood

The results are expressed in milliequivalents per liter of blood.

Species	No. of subjects	Source of blood	Method	Buffer alkali			
				Mean	Range	s	s'
Human...	31	Vein	Macro	37.5	35.1-41.2	1.55	0.3
" ...	31	Finger-tip	Micro	37.4	34.9-41.4	1.65	0.8
Rat....	24	Tail	"	37.8	35.8-40.7	1.27	0.8

s is the standard deviation of the distribution $= \sqrt{d^2/(n-1)}$. s' is the standard deviation of variations from individual means.

necessary to establish significance is 2.7 (by extrapolation from the value for $n = 30$ to $n = 38$ (Fisher (43) p. 139)).

Normal values for man and for the white rat, together with estimates of variance, are summarized in Table VII. The standard deviation of variations from individual means, s' , is given in terms of milliequivalents rather than as the usual percentage deviation, because the latter depends on the absolute value of the buffer alkali, while the former tends to be constant over a wide range.

The rats used to obtain the data in Table VII were 3 to 4 months old and had been on a constant diet of Purina Dog Chow after being weaned. The observed values cover approximately the same range as normal human blood. In order to ascertain whether the slight struggling incidental to the collection of blood

from the tail influences the results, samples were taken from seven rats before and during urethane anesthesia (2 gm. per kilo by mouth). The results were not significantly different. Smith and Smith (44) found that urethane anesthesia did not influence the electrolyte pattern of rat serum.

SUMMARY

A method, based on that of Greenwald and Lewman, and designed for the rapid estimation of the buffer alkali in cutaneous blood, is described. An effect of dilution is pointed out and possible causes are considered. There remains an unexplained dilution difference of about 3 milliequivalents per liter of whole blood.

Comparison of the base bound by the individual buffers in the arterial blood of normal men with the buffer alkali measured at a 1:10 dilution indicates that the latter yields essentially correct values. This conclusion is also supported by the results of an estimate of the total available base by summation of the buffer alkali and the base bound by the phosphate esters of the red cells. In this connection, a titration curve of diphospho-1-glyceric acid is given.

I am indebted to Professor I. Greenwald for suggesting this problem and for his advice and criticism throughout the course of the work.

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THE INFLUENCE OF CORTIN AND SODIUM CHLORIDE ON CARBOHYDRATE AND MINERAL METABOLISM IN ADRENALECTOMIZED DOGS

By EDWARD C. KENDALL, EUNICE V. FLOCK, JESSE L. BOLLMAN,
AND FRANK C. MANN

*(From the Division of Biochemistry and the Division of Experimental
Medicine, The Mayo Foundation, Rochester, Minnesota)*

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In the normal dog insulin, epinephrine, or an injection of a solution of glucose causes a marked decrease of the concentration of phosphate and potassium ions in the serum (3, 4). The close relation between carbohydrate and mineral metabolism indicated by these results suggested further investigation of the influence of carbohydrate metabolism on the distribution and excretion of inorganic ions in adrenalectomized dogs.

The intravenous injection of a solution of 10 per cent glucose into normal dogs produced a mild diuresis without glycosuria; the excretion of potassium varied but was usually at a faster rate than before the injection. In no instance did the injection produce weakness, prostration, or other toxic manifestation. The high blood sugar during the injection promptly decreased to hypoglycemic levels within a few minutes after the injection was stopped, and the concentration of the potassium and phosphate in the serum returned approximately to their former values within 2 or 3 hours.

Adrenalectomized dogs that had been maintained in good condition with cortin or with the administration of sodium chloride and sodium citrate were injected for 3 hours with a solution of 10 per cent glucose at the rate of 1 gm. per kilo per hour. They responded as normal dogs, provided the interval between the last administration of cortin or sodium salts and the injection of glucose was but a few hours.

If the interval between the last administration of cortin and the

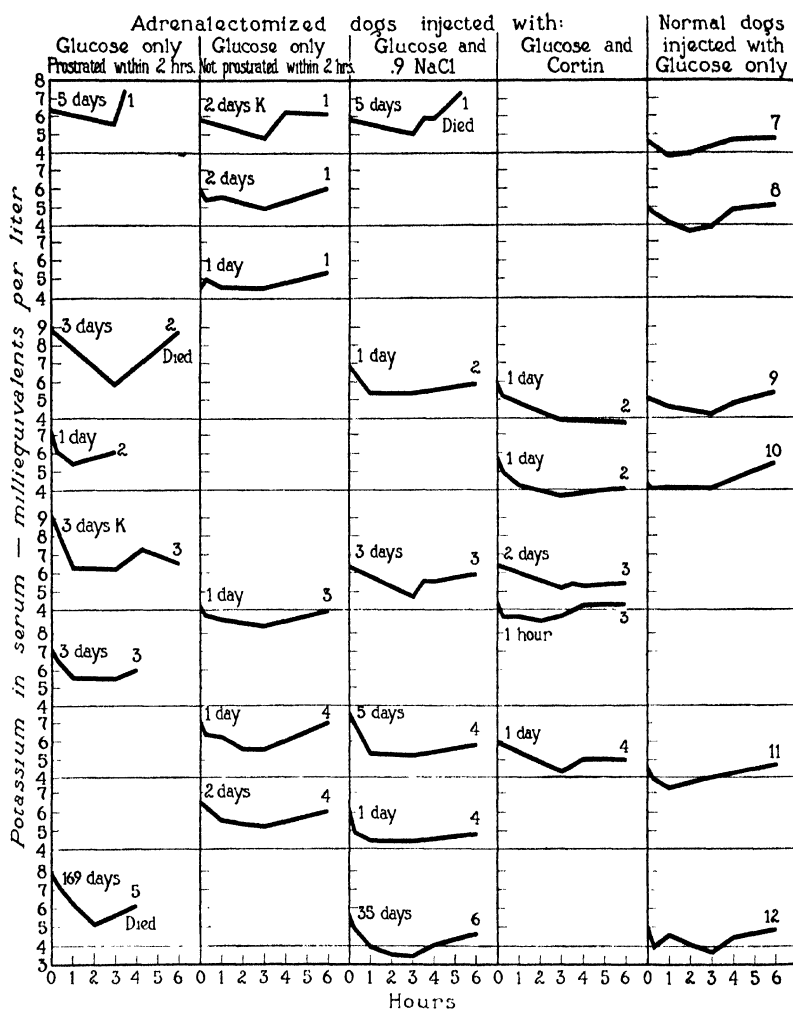


FIG. 1. Curves are labeled with the number of the dog at right and interval in days since the last administration of cortin at left. Curves may be identified by number of column and by position of curve numbered from the top. In Columns 1 and 2 "prostrated" and "not prostrated" refer to the condition 2 hours after the end of the injection.

Column 1, Curve 2. Treatment with 50 cc. of 5 per cent sodium chloride, 50 cc. of 20 per cent glucose, and 15 cc. of cortin given at 3.5 hours. At 6 hours 50 cc. of 5 per cent sodium chloride and 25 cc. of 20 per cent glucose

injection of glucose was as long as 5 days, the result was strikingly modified by the effect of cortico-adrenal insufficiency which had developed while the cortin was withheld. During the interval

were given. The dog died at about 9 hours. Curve 4. The dog received 1 gm. of potassium as dipotassium phosphate in the diet on each of 3 days during which cortin was withheld. At 4.5 hours the dog was completely prostrated; 10 cc. of cortin, 100 cc. of 5 per cent sodium chloride, and 45 cc. of 20 per cent glucose were given. The dog made an uneventful recovery. Curve 6. The dog had been maintained 169 days without cortin but with 6 gm. of sodium chloride, and 5 gm. of sodium citrate added to the diet, and 0.5 per cent sodium chloride in the drinking water. The daily diet contained 0.8 gm. of potassium. The dog died without treatment 1 hour after injection was stopped. Compare with Column 3, Curve 6. This dog had been maintained 35 days without cortin but with 10 gm. of sodium chloride, 5 gm. of sodium citrate in the diet, and 0.6 per cent sodium chloride in the drinking water. For the preceding 14 days the diet contained 350 mg. of potassium.

Column 2, Curve 5. The dog had convulsions simulating those associated with hypoglycemia. The blood sugar was 25 mg. per cent; serum potassium 6.9 milliequivalents per liter. Curve 6. The dog was in estrus, which may have affected the response to the injection. The dog did not have convulsions.

Column 3. All the dogs responded as did normal dogs except in the experiment represented in Curve 1. Anuria developed at the end of the injection. At 4 hours, 1 hour after the injection was stopped, the dog was completely prostrated and was given 10 cc. of cortin, 65 cc. of 5 per cent sodium chloride, and 0.5 cc. of 1:1000 epinephrine. At 5 hours the dog was nearly dead and was given sodium chloride, cortin, and glucose, but without effect; the dog died 15 minutes later.

Column 4, Curve 1. Glucose and cortin only were given. Curve 2. Glucose and 100 cc. of cortin with 0.9 per cent sodium chloride were given. The injection produced glycosuria; 2.66 gm. of glucose were excreted.

Column 5, Curve 1. The dog received 25 cc. of cortin with the glucose; all the other dogs received glucose only. No glycosuria was produced.

The rise in the concentration of potassium during the interval when cortin was withheld is shown by the series of injections on Dogs 1, 2, 3, and 4 during various stages of adrenal insufficiency. Dog 3 (Curve 4, Columns 2 and 4) was in excellent condition and was the only dog which reacted as normal when injected with glucose alone. Glucose alone after 5 days without cortin (Column 1, Curve 1) produced a marked prostration associated with anuria but vigorous treatment revived the dog. The injection of glucose with sodium chloride was tolerated in the experiment represented in Column 3, Curve 4, but not in the experiment represented in Column 3, Curve 1. Failure to restore renal function was associated with a rapid and continuous rise in the concentration of potassium in the serum.

after the administration of cortin was stopped and the time of the injection of glucose there was a continuous increase in the concentration of potassium and urea in the serum.

Without exception the injection produced a decrease in the concentration of potassium in the serum, but the lowest value reached was higher than the corresponding value in normal dogs. Instead of a diuresis there was suppression of urine. Soon after the injection of glucose was stopped, the concentration of potassium in the serum increased to a higher value than in the normal dog, and associated with the increase in the concentration of potassium were weakness, prostration, and death unless vigorous treatment was instituted. The injection of a solution of 5 per cent sodium chloride, glucose, and cortin usually was sufficient to overcome the prostration, and after a few days the dog was restored to a normal condition.

Varying degrees of cortico-adrenal insufficiency were produced by changes in the length of the interval between the last administration of cortin and the injection of glucose. Marked differences in the rate at which cortico-adrenal insufficiency developed were observed among five dogs. With one dog (Column 1, Curve 3, Fig. 1) 24 hours without cortin were sufficient to produce as marked a deficiency as that which followed an interval of 3 to 5 days without cortin in four other dogs.

When a solution of 10 per cent glucose which also contained 0.9 per cent sodium chloride was injected for 3 hours at the rate of 1 gm. per kilo per hour into adrenalectomized dogs, they behaved as normal dogs. In each of six such experiments the injection decreased the concentration of potassium in the serum. Associated with five of the six injections there were diuresis and excretion of potassium and the increase of potassium in the serum after the injection was stopped was neither rapid nor to a high value. In one of the dogs (Column 3, Curve 1) which had not been given cortin for 5 days previous to the injection, renal function was impaired and could not be restored. The dog died 3.5 hours after the injection was stopped.

When cortin was given with the injection of glucose but without sodium chloride, the adrenalectomized dogs reacted as normal dogs. The diuresis and excretion were even more marked and the increase in the concentration of potassium after the injection was

stopped was both less rapid and to a lower level than in the normal dog. Five such injections into three dogs gave uniform results. In one dog to which large amounts of cortin were given glycosuria was produced.

Three of twenty-three injections of 10 per cent glucose into adrenalectomized dogs terminated fatally. The results with one have been described; the other two dogs were both injected with a solution of 10 per cent glucose without sodium chloride or cortin. In one of the dogs (Column 1, Curve 2) the injection decreased the potassium in the serum but it rapidly increased when the injection was stopped. There was complete suppression of urine which continued even after an intravenous injection of sodium

TABLE I

Blood Urea, in Mg. Per Cent, in Dogs at Time of Injection of 10 Per Cent Glucose Solution at Rate of 1 Gm. per Kilo per Hour

Curve No., position from top of column	Column No.			
	1	2	3	4
1	74	60	96	44
2	86	54	48	34
3	42	62	56	76
4	138	46	60	46
5	89	41	46	50
6	64	54	24	

chloride and cortin. There were 12.7 milliequivalents per liter of potassium in the serum at the time of death.

The third dog (Column 1, Curve 6) had been maintained with sodium chloride and sodium citrate but without cortin for 169 days prior to the injection of glucose. 2 hours after the start of the injection the concentration of potassium in the plasma had decreased but 1 hour after the injection was stopped the dog became prostrated and died within 30 minutes without treatment. At the time of death the potassium in the serum was 6.1 milliequivalents per liter.

Blood Urea—The values for the blood urea at the time of the injection of glucose are given in Table I. The blood urea, together with the number of days during which cortin was withheld, furnishes information in regard to the degree of cortico-adrenal

insufficiency, although the concentration of urea was quite unrelated to the response of the dogs to the injection of glucose.

Blood Sugar—The initial blood sugar of the dogs used in the twenty-three injections represented in Fig. 1 ranged from 51 to 105 mg. per cent: two were above 80 and five were below 60 mg. per cent. The injection of glucose in each dog caused an increase in the blood sugar: one was above 400, two were between 300 and 400, eight were between 200 and 300, and seven between 100 and 200 mg. per cent. The blood sugar 1 hour after the injection was stopped was below normal in all experiments except that represented in Column 1, Curve 6. During the injection the blood sugar in this dog rose to 488 mg. per cent and 1 hour after the injection was stopped it was 230 mg. per cent. The blood sugar in the other experiments, expressed in mg. per cent, was between 20 and 30 in six, 30 and 40 in nine, 40 and 50 in six, and was 59 in the experiment represented in Column 4, Curve 4. The blood sugar remained low in only one experiment, shown in Column 2, • Curve 5. 3.5 hours after the injection was stopped, the dog was in convulsions simulating those of insulin shock. This is the only experiment in which there were convulsions. The blood sugar was not associated with the severity of the weakness and prostration which were produced by the injection of glucose. In some experiments the blood sugar returned promptly to normal levels and in others it remained low for 2 or 3 hours but the prostration was not always associated with the low level of blood sugar and in many instances there was a low level of blood sugar without weakness or other symptoms.

Sodium and Chloride in Serum—The injections of glucose with and without 0.9 per cent sodium chloride did not cause a change in the concentration of sodium and chloride beyond normal limits. In twenty of the twenty-three experiments the highest concentrations of sodium in the serum were between 135 and 148 milliequivalents per liter. In two experiments they were slightly above and in one slightly below these values. The lowest concentrations of sodium in the serum were between 126 and 143 milliequivalents per liter for twenty of the experiments; in one it was slightly higher and in two slightly lower.

Variations of the same order were shown for the concentration of the chloride ion. In twenty experiments the highest values

were between 107 and 120 milliequivalents per liter, with three slightly below. The lowest values for twenty experiments were between 98.6 and 110 milliequivalents per liter, with one lower and two higher.

The concentration of sodium and chloride in the serum was quite independent of the changes in urea, blood sugar, potassium, phosphates, or any symptoms of cortico-adrenal insufficiency.

Concentration of Phosphate in Serum—In twenty-two of the twenty-three experiments the injection of glucose with or without sodium chloride or cortin caused a drop in the concentration of the phosphate ion which was followed in eighteen experiments by an increase in the level of phosphate after the injection was stopped. No determinations were made in the other four experiments but there is every indication that the phosphate consistently increased after the injection was stopped. The one experiment in which the phosphate did not decrease is represented in Column 1, Curve 3. Since the blood sugar was lower in this dog during the injection than in any other dog, there is no doubt that the utilization of glucose was rapid, and the most probable explanation for the failure to demonstrate a change in the phosphate is the fact that it was low, 2.3 mg. per cent, at the beginning of the injection. After the injection was stopped, there was an increase in the serum phosphate to 2.8 mg. per cent. The changes in the phosphate ion in the serum were associated with the decrease and increase in the potassium ion which were produced by the injection of glucose.

Osmotic Pressure of Serum—Through the kindness of Dr. Roepke the osmotic pressure of the serum in thirteen of the experiments was determined by the method of Baldes (2). There were no consistent changes associated with the degree of cortico-adrenal insufficiency. The highest values were in the experiments represented in Column 1, Curve 2, and Column 4, Curve 1. In each of these an osmotic pressure equivalent to 1020 mg. per cent of sodium chloride was determined. The lowest value, equivalent to 890 mg. per cent of sodium chloride, was found in the dogs that were injected with glucose and 0.9 per cent sodium chloride.

Hematocrit Reading—Determinations of the hematocrit reading were made in thirteen of the twenty-three experiments. The lowest values, from 36 to 41 per cent, were found in the dogs injected with glucose alone and in which anuria was produced.

The highest readings, from 57 to 63 per cent, were found in the experiment represented by Column 1, Curve 2. This dog died, but in other experiments with almost as high a value, 58 and 59 per cent (Column 1, Curves 1 and 4), the dogs became prostrated but were restored to normal with sodium chloride, cortin, and glucose. As would be anticipated from the work of Swingle and his coworkers (10), the hematocrit reading was highest in the dogs with the most marked cortico-adrenal deficiency but it is clear that the cause of the weakness and prostration is not an increase in the value of the hematocrit reading. The injection of glucose caused a slight decrease in the hematocrit reading and the value after the injection was stopped was but a little higher than that at

TABLE II

Total Amount of Sodium and Potassium Excreted in Urine during 6 Hours after Beginning of 3 Hour Injection of Glucose and Cortin

Dog No.	Weight	Days off cortin	Volume of urine	Sodium	Potassium	Cortin
	<i>kg.</i>		<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>cc.</i>
2	15.6	1	213	1020	546	30
2	15.5	1	863	1324	1123	100*
3	14.2	2	137	48	396	25
3	16.0	0	433	391	392	23
4	14.2	1	332	82	502	25
4	13.8	5	210	1760	495	0*
3	15.0	3	149	591	292	0*

* 0.9 per cent sodium chloride was added to the solution of glucose.

the beginning of the experiment. This increase was accompanied by a decrease in the osmotic pressure of the serum.

Excretion of Sodium and Potassium by the Kidney—Throughout these experiments the urine which was passed was collected at hourly intervals. The determination of sodium and potassium was of little significance if only 1 or 2 hourly samples were given by the dog because of the development of anuria. The experiment represented in Fig. 1, Column 2, Curve 4, is the only one of the first thirteen experiments whose results are shown in this figure during which anuria did not develop. It is difficult to interpret the influence of the injection of sodium chloride on the rate of excretion of sodium and chloride ions but the results of two

experiments are given for comparison. The dogs were injected with 10 per cent glucose at the rate of 1 gm. of glucose per kilo of body weight per hour for 3 hours. The curves representing the potassium in the serum can be found in Fig. 1 from the number of the dog and the days during which cortin had been withheld (Table II).

Methods

The methods used for the determination of the constituents of the blood and urine have all been given in a recent paper (3). The determination of the osmotic pressure was made with the method of Baldes.

DISCUSSION

The injection of glucose for 3 hours with and without sodium chloride or cortin produced in each experiment a decrease in the concentration of potassium in the serum. As long as there was active utilization of glucose, the potassium remained at the lower level but soon after the injection of glucose was stopped the concentration of potassium in the serum increased. Normal dogs and adrenalectomized dogs treated with cortin or sodium chloride and sodium citrate were not affected by these changes but adrenalectomized dogs not treated with cortin or sodium salts became progressively more sensitive to the condition produced by the cessation of a continuous injection of glucose.

Both sodium chloride and cortin favorably influenced the excretion of potassium by the kidney (1, 5). The effect of sodium chloride and of cortin is without doubt exerted throughout all the tissues of the body but the important position of the kidney for the elimination of potassium should be pointed out (6, 8). The influence of cortin on the excretion of potassium was striking.

In eleven of twelve injections when a 10 per cent solution of glucose alone was given to adrenalectomized dogs, suppression of urine occurred and in some dogs anuria persisted for several hours after the injection was stopped. In these dogs the concentration of potassium in the serum was higher than that of the dogs with diuresis and the only dogs which became prostrated and died were those with anuria. Improvement in the condition of the dog after the injection of sodium chloride and cortin was associated with the return of active renal function.

The one adrenalectomized dog that did not develop anuria during the injection of 10 per cent glucose (see Column 2, Curve 4) had received treatment with cortin except for the preceding 24 hours. When cortin was withheld for 3 days before glucose was injected, the urine was completely suppressed and the dog became prostrated and continued in a critical state for several hours before renal function was restored. The injection of sodium chloride, cortin, and glucose followed by a transfusion relieved the prostration and the dog made an uneventful recovery.

We have shown (8) that crystalline cortin-like Compounds A and B which were separated in this laboratory from the adrenal cortex possess qualitatively the physiologic properties of the "salt and water hormone" and the recent work of Long and his associates (9) has shown these same compounds to be diabetogenic in the partially depancreatized rat. The proof that the "salt and water hormone" and the agent which produces glycosuria are identical emphasizes the importance of mineral metabolism on the utilization of carbohydrate. In adrenalectomized dogs the essential chemical changes associated with carbohydrate metabolism can proceed without the "salt and water hormone" of the adrenal cortex, provided an adequate supply of sodium and chloride ions is available. In partially depancreatized rats, however, sodium chloride does not produce glycosuria (9).

The probable influence of inorganic ions such as sodium and chloride is through osmosis, ion antagonism, and changes in the permeability of membranes, but how these same effects can be produced by cortin-like compounds has not been shown.

The results of the injections of glucose which have been described indicate that in the absence of cortin or adequate amounts of sodium and chloride the experimental animal becomes highly sensitive to potassium. It therefore seems probable that cortin modifies the rate of some chemical change which involves potassium. When this chemical change fails, control of potassium metabolism also fails and the toxic effects of the potassium ion become evident. It should be emphasized, however, there is no definite concentration of potassium in the serum which is toxic. The essential chemical change which involves potassium metabolism may fail and cause death without a marked rise in the concentration of potassium in the serum. On the other hand, Keith

and Binger (7) have shown that the concentration of potassium may be raised to 11 milliequivalents per liter in the serum of normal persons without toxic effects.

The effect of cortin on the excretion of potassium was clear and uniform: the largest amount of potassium was excreted when the largest amount of cortin was used. The effect of cortin on the excretion of sodium was not uniform. The difference in the excretion of sodium in the two experiments on Dog 3 when cortin was used may be due to the difference in the interval after the last administration of cortin. When 100 cc. of cortin were added to the injection of Dog 2 the excretion of sodium was much greater than in Dog 3 without cortin, but the excretion of sodium in Dog 2 was less than in Dog 4, which did not receive cortin. Sodium chloride, 0.9 per cent, was added to the solution of glucose in all three of these experiments. There is a difference in the time during which cortin was withheld from these three dogs, which is probably an important factor.

SUMMARY

The influence of carbohydrate metabolism, cortin, and sodium chloride on potassium metabolism has been studied during the intravenous injection of a solution of 10 per cent glucose into adrenalectomized dogs for 3 hours at the rate of 1 gm. per kilo of body weight per hour. The injection of glucose with or without cortin or sodium chloride decreased the concentration of potassium in the serum. When either cortin or 0.9 per cent sodium chloride was added to the 10 per cent solution of glucose, there was a mild diuresis without glycosuria but potassium was readily excreted by the kidney. The injection of glucose alone at the same rate produced anuria.

During each injection the blood sugar was high but after the injection was stopped the concentration of glucose usually decreased to hypoglycemic levels. The decrease in the active utilization of sugar was followed by an increase in the concentration of potassium in the serum. Associated with the increase of potassium in the serum there were weakness and prostration only in the adrenalectomized dogs that had been deprived of cortin for from 1 to 5 days. Anuria developed in each of these dogs during or immediately following the 3 hour injection of glucose. After the

injection of glucose was stopped, weakness and prostration did not appear in any of the dogs in which there had been a diuresis.

In these experiments sodium chloride appeared to be the full equivalent of cortin in respect to the excretion of potassium and the response of the adrenalectomized dog to an injection of glucose.

The disposal of injected glucose can occur to the same extent and is associated with the same changes in the concentration of potassium and phosphate in the serum in adrenalectomized as in normal dogs.

Of the constituents of the blood serum, only the concentration of potassium could be correlated with the symptoms of cortico-adrenal deficiency. The concentration of phosphate increased and decreased with the concentration of potassium but no consistent changes were found in the serum of sodium or chloride concentration, the osmotic pressure, or the hematocrit reading.

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A BALANCE SHEET OF FAT ABSORPTION*

I. THE STORAGE OF ELAIDIC ACID BY THE RAT OVER A ONE DAY PERIOD

By MATHIAS F. F. KOHL

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

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Sinclair, in 1935, fed elaidin to rats and found that elaidic acid replaces part of the normal phospholipid fatty acids of muscle and liver. The rates of entrance of elaidic acid into and disappearance from phospholipid are rapid in liver and slow in muscle (1). Sinclair also found elaidic acid in the phospholipids of the blood plasma of cats during elaidin lipemia. He believes that plasma phospholipid serves as a mechanism for the transportation of elaidic acid and therefore of ordinary fatty acids to the sites of utilization (2). The presence of elaidic acid in the phospholipids of the intestinal mucosa of the cat during the absorption of elaidin has also been shown by Sinclair and Smith (3). McConnell and Sinclair have reported the entrance of small amounts of elaidic acid into the phospholipids of rat brain (4). Haven has observed that the turnover of elaidic acid in the phospholipid of rat tumor, like that in muscle phospholipid, is a slow process (5). These studies indicate that further information may be gained concerning the metabolism of the fatty acids by the use of this unnatural isomer of oleic acid. In this investigation balance sheet studies of fat absorption, with elaidic acid as an indicator, have been carried out.

* These studies are taken from a dissertation submitted to The University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy, February, 1938. Part of this investigation was presented before the National Academy of Sciences at Rochester, New York, October 27, 1937 (Kohl, M. F. F., *Science*, **86**, 447 (1937)).

EXPERIMENTAL

Male rats, of Wistar strain, were raised from weaning on a calf meal diet formulated by Maynard (6) until a weight of 100 ± 5 gm. was reached. The rat was then fasted 15 hours to insure a postabsorptive state, after which a known amount of food was placed in the food cup and smooth paper placed under the cage to collect spilled food. Only those animals **were** used that ingested sufficient food, especially during the beginning of the feeding period, to insure an immediate maximum absorption. At the end of the feeding period the uneaten food along with the small amount that was usually spilled was dried in a desiccator and weighed. The amount ingested by the rat was obtained by difference. In one series of experiments elaidin was the sole constituent of the diet. In another series the diet used was No. 262 formulated by Sinclair (7) in which 40 per cent of the calories of the diet is furnished by elaidin, 34 per cent by cane-sugar, 18 per cent by casein, and 8 per cent by yeast.

After the experimental absorption period the animal was sacrificed. The procedures followed in extracting and separating the lipids of the various tissues were essentially those used by Bloor (8) and Sinclair (9, 10). The acetone-soluble (neutral fat and cholesterol) and the acetone-insoluble (phospholipid) lipids of liver and carcass¹ were saponified and the fatty acids isolated and weighed. Iodine numbers were determined by Yasuda's modification of the Rosenmund-Kuhnhehn pyridine sulfate dibromide method (11).

The procedure used for the determination of elaidic acid in the solid fatty acids was based on the microprocedure outlined by Sinclair for the separation of solid from liquid acids (1, 10). The method was modified to permit the separation of amounts of mixed fatty acid varying from 100 to 25 mg. 9 cc. of alcohol per 100 mg. of mixed fatty acid were used for the first separation and 5 cc. per 100 mg. for the second. The solid fatty acids isolated were washed free from acid and lead, dried with Na_2SO_4 , weighed, and the iodine number determined. Weights less than 10 mg. were checked by the Bloor oxidative method. The presence of small amounts of liquid unsaturated acids in the solid acids iso-

¹ The term "carcass" is used to designate the body minus the liver and gastrointestinal tract.

lated is a difficulty that cannot be readily surmounted in the micro- or semimicrofractionation of liquid and solid acids. These so called "control" iodine numbers of the solid fatty acids of various tissue lipids in the rat obtained with our method are of the same magnitude as those obtained by the Sinclair procedure. The average values for determinations on ten young rats raised on the calf meal diet are: carcass total lipid fatty acids 7 ± 1 ,² carcass neutral fat fatty acids 7 ± 1 , carcass phospholipid fatty acids 8 ± 1 , liver phospholipid fatty acids 5 ± 1 , liver neutral fat fatty acids 8 ± 2 . The elaidic acid in a given amount of fatty acid is estimated by multiplying the weight of the solid acids isolated by their iodine number, corrected for the control value, and then dividing by 90.

To determine unabsorbed elaidic acid the gastrointestinal tract was freed of as much mesentery as was possible without tearing through the thin wall and split with forceps and probe in a large beaker. The ground up feces of the experimental period were also added. The unabsorbed fat was extracted with successive portions of cold ethyl ether.³ A small correction had to be made for the fat of the adhering mesentery and the tissue lipids extracted by the solvent. This was done by determining the liquid acids present in the isolated fatty acids. Since the ratio of liquid to solid acids in normal rat fat was determined to be 72:28, the solid acids associated with these tissue liquid acids can be calculated. The sum of these two contaminating fractions subtracted from the total fatty acid isolated gives the unabsorbed elaidin fatty acid. The iodine number of the solid fatty acids also served as a check on the amount of contaminating tissue saturated acids in the elaidin fatty acids. In two experiments in which 100 mg. of elaidin were added to gastrointestinal tracts of rats in the postabsorptive state the recoveries of elaidin were 99 and 101 mg. respectively.

² The average deviation is used to express the experimental variation throughout this study.

³ Cold alcohol was used at first but was found to have a greater solvent effect on the tissue lipids than ether. In Experiment II of these studies, in order that the amount of elaidic acid in the phospholipids of the gastrointestinal tract could be determined, the lipids of the tract were extracted quantitatively with hot alcohol. The unabsorbed elaidin fatty acids were determined in the acetone-soluble lipids. This fraction consisted almost entirely of diet fat.

The method used in the preparation of elaidin by treatment of olive oil with N_2O_5 was that of Sinclair (1, 2). The fatty acids of two batches prepared had iodine numbers of 72 and 74. No liquid acids could be detected. The fatty acids of the two lots of elaidin contain about 80 and 82.2 per cent elaidic acid, respectively, the remaining acid undoubtedly being palmitic acid. Inasmuch as this saturated acid cannot be traced in the body, the balance sheet represents approximately 80 per cent of the fat fed.

In Experiment I elaidin was fed *ad libitum* over a 1 hour period, the time of the initial eating of food being taken as the beginning of the absorption period. Twenty-four animals were used in determining the absorption curve of elaidic acid during the following 24 hour period. The tissues of fifteen of these rats were also analyzed for elaidic acid. In Experiment II Diet 262, containing elaidin as 40 per cent of the calories, was fed. Sixteen animals were used to determine the absorption and storage curves of elaidic acid during the first 24 hours of feeding. The rats were permitted to eat *ad libitum* Diet 262 throughout the entire period. The fatty acids of the elaidin fed in both experiments had an iodine number 72. The data for absorption and storage of elaidic acid in Experiment I are presented in Fig. 1. Fig. 2 shows the partition between the various tissue lipids of the elaidic acid found in the bodies of typical animals chosen from Experiment I. Data for Experiment II are given similarly in Figs. 3 and 4.

DISCUSSION

In Experiment I (Fig. 1) a lag in absorption prior to the 9th hour is evident. This is undoubtedly due to the solid nature of the elaidin which must be liquefied prior to digestion and absorption. The absorption curve is linear despite the fact that the amount of diet fat remaining in the digestive tract is markedly lowered toward the end of the absorption period. Interestingly enough, Cori has shown that the absorption of glucose by the rat proceeds at a constant rate that is independent of the concentration of the solution given or the absolute amount of sugar in the gastrointestinal tract (12).

An absorption lag is not present when protein and carbohydrate are fed along with the fat in Experiment II (Fig. 3). It was found in this series of animals that the liquefaction of elaidin was accom-

plished with considerable ease, since the solid particles of fat found in the stomach were small and few in number as contrasted to the large solid lumps found during the early course of the absorption period when elaidin alone was fed. The liquefaction of elaidin in Diet 262 is greatly influenced by the fine dispersion of the fat par-

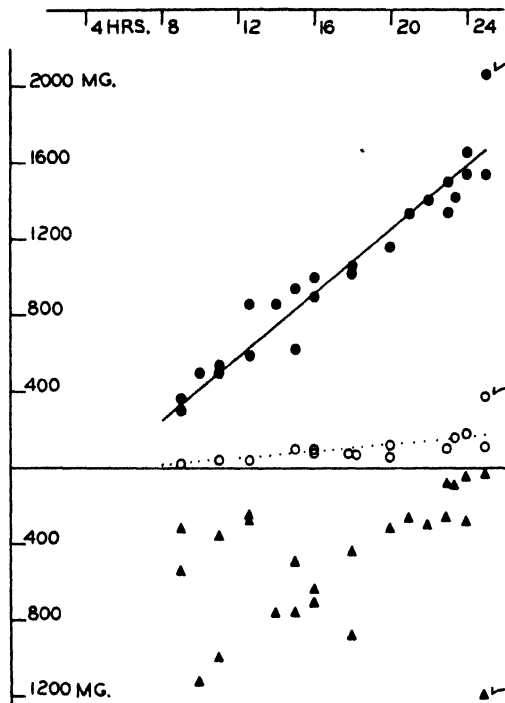


FIG. 1. Experiment I. Rats fed elaidin over a 1 hour interval (except in the experiment indicated by checks, in which feeding was for 3 hours). The elaidin fatty acids contained 80 per cent elaidic acid. Unabsorbed elaidic acid is indicated as ▲, absorbed elaidic acid as ●, and elaidic acid found in the tissues as ○.

ticles throughout the diet mixture, which prevents the fat from lumping, thus cutting down surface. That the emptying time of the stomach may also be shortened by the dilution of the fat with protein and sugar must also be considered. The absorption curve of elaidic acid in this experiment is linear. Since the animals ate

ad libitum, copious amounts of fat were present in the gastro-intestinal tract throughout the entire period.

In Experiment I the amount of the absorbed elaidic acid found in the tissues is only a small part of that absorbed. When the rates of absorption and deposition of elaidic acid are calculated from the slopes of the experimental curves in Fig. 1, it is found that elaidic acid is absorbed at the rate of approximately 80 mg. per hour, of which 8 mg. are found in the tissues and 72 mg. have

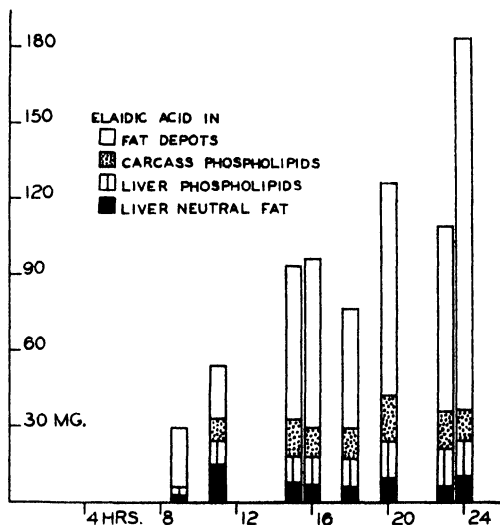


FIG. 2. The partition of the elaidic acid found in the bodies of typical animals chosen from Experiment I (Fig. 1). The total tissue elaidic acid of the animal is represented by the entire bar and the elaidic acid in the various tissue lipids by the designated bars that make up the whole.

disappeared. Large amounts of elaidic acid are found in the tissues in Experiment II despite the fact that the absorption rate is lower than that in Experiment I. The calculated absorption rate is approximately 61 mg. of elaidic acid per hour, of which 26 mg. find their way into the tissues and 35 mg. disappear. It is evident that fat is absorbed less rapidly when fed with protein and sugar than when fed alone. Irwin and others have reported similar data (13).

The depot fat content of animals in Experiments I and II was

2.66 ± 0.77 and 2.88 ± 0.68 gm. respectively. A control value established on ten male rats raised on calf meal and weighing 100 ± 3 gm. was 2.45 ± 0.74 gm. of fatty acid. Thus the two experiments show a gross positive fat balance. Since the depots were in a state of positive balance, it appears that the fat absorbed during the experimental period exceeded the demand of the tissues for fat to supply metabolic needs. The following conclusion appears reasonable. The rat in the postabsorptive state

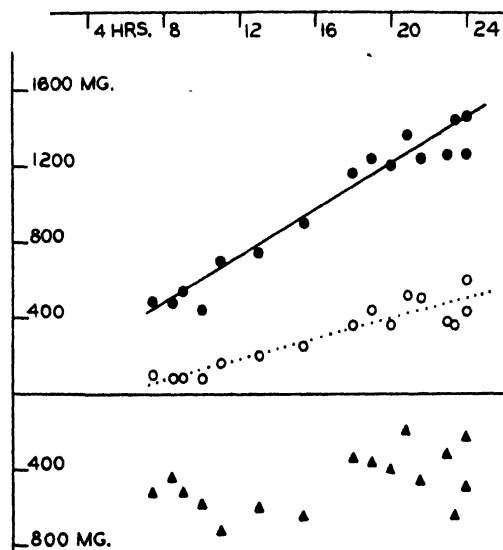


FIG. 3. Experiment II. Rats fed *ad libitum* Diet 262 which contains elaidin as 40 per cent of its calories. The elaidin fatty acids contained 80 per cent elaidic acid. Unabsorbed elaidic acid is indicated as ▲, absorbed elaidic acid as ●, and elaidic acid found in the tissues as ○.

is burning fat which is being discharged from the depots into the blood in response to some as yet unknown metabolic stimulus. With the inflow of fat into the blood from the intestine following the feeding of elaidin, active mobilization of depot fat ceases and the absorbed fat is catabolized. A regulatory mechanism that stops the flow of fat from the depots when absorption of fat begins and stores the exogenous fat from the intestine that accumulates in excess of the tissue needs is suggested. In Experiment II,

in which the diet is normal in respect to protein and sugar, the elaidic acid deposited in the tissues is greatly increased despite the fact that the amount absorbed is decreased. This is in keeping with the concepts of Pettenkofer and Voit (14) who proposed that fat is used in the vital economy to make up an energy deficit that remains after available protein and sugar have been utilized. The fat in excess of this need is stored as a reserve.

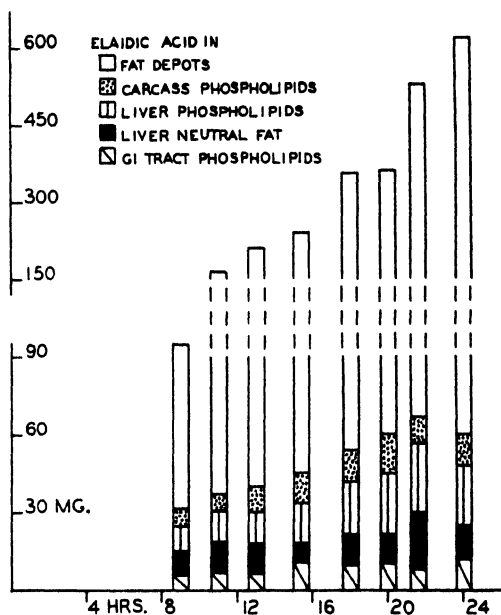


FIG. 4. The partition of the elaidic acid found in the bodies of typical animals chosen from Experiment II (Fig. 3). The total tissue elaidic acid of the animal is represented by the entire bar and the elaidic acid in the various tissue lipids by the designated bars that make up the whole.

In Experiment I (Fig. 2) small amounts of elaidic acid are found in the liver neutral fat⁴ during the absorption period. The entrance of elaidic acid into the phospholipid of the liver is also

⁴ This fraction includes the fatty acids of both liver neutral fat and cholesterol esters but for convenience is designated as neutral fat. Whether or not elaidic acid enters into the cholesterol esters of the liver has not been determined.

prompt. This elaidic acid makes up 22 ± 5 per cent of the total neutral fat fatty acids and 12 ± 2 of the phospholipid fatty acids of the liver. The amount of elaidic acid entering the phospholipids of the carcass is of a magnitude similar to that found in the liver, yet the amount is only 1.6 ± 0.5 per cent of the fatty acids in the carcass phospholipids. This clearly indicates the difference in the rates of uptake of elaidic acid by phospholipid of carcass and liver as previously described by Sinclair (1). The depot elaidic acid comprises the greater part of that found in the tissues. The balance sheet for Experiment I is lacking in respect to the entrance of elaidic acid into the phospholipids of the intestinal mucosa during absorption.

In Experiment II (Fig. 4) the per cent elaidic acid in the phospholipid fatty acids of the entire gastrointestinal tract rises from 7 per cent at the 9th hour to approximately 15 per cent by the 24th. Since it is believed that this entrance of elaidic acid takes place primarily into the phospholipids of the mucosa of the small intestine, which in this experiment are diluted by the phospholipids of the other tissues of the tract, the per cent of elaidic acid in the mucosal phospholipids would be quite high. Elaidic acid is also found in the liver neutral fat fraction to the extent of 24 ± 2 per cent of the total fatty acids. The elaidic acid in the liver phospholipid fatty acids rises from 9 to 18 per cent from the 9th to the 24th hour, again indicating the rapid entrance of elaidic acid into the liver phospholipids. Elaidic acid enters the carcass phospholipids slowly, to the extent of 1.7 ± 0.4 per cent. The elaidic acid entering the fat depots increases rapidly as the absorption period continues, representing fat absorbed in excess of the immediate need.

In comparing Figs. 2 and 4, one notices in the latter a larger deposition of elaidic acid in the depots. This has been discussed previously. The amount of elaidic acid entering the carcass phospholipids is approximately the same in the two experiments. However, in regard to the entrance of elaidic acid into the liver a distinct difference is noted. The livers of ten control animals averaged 5.18 ± 0.75 gm. and contained 125 ± 14 mg. of phospholipid fatty acid and 29 ± 5 mg. of neutral fat fatty acid. In Experiment I, in which the diet was deficient, the livers conse-

quently decreased in size to 4.45 ± 0.55 gm. with a phospholipid fatty acid content of 101 ± 12 mg. In Experiment II, in which the animals were continued on an essentially normal ration, the phospholipid fatty acid content of the liver was the same as that of the control animals; namely, 123 ± 10 mg. In Experiment I the elaidic acid replaces an average of 12 per cent of the phospholipid fatty acids, while in Experiment II the replacement increases from 9 to 18 per cent during the absorption period studied. The amounts of elaidic acid in the liver neutral fat are slightly greater in Experiment II than in Experiment I. The total fatty acids of the liver neutral fat in the two experiments are 41 ± 11 and 33 ± 7 mg. respectively, with elaidic acid contents of 24 and 22 per cent. This difference is not remarkable.

SUMMARY

Balance sheet studies on the absorption of elaidic acid carried out on young rats over hourly periods after feeding elaidin as the sole food source show that elaidic acid is absorbed at a nearly constant rate until absorption is practically complete, following an initial lag period required for the liquefaction and digestion of the solid fat. Only a very small amount of the absorbed elaidic acid is found in the tissues, the remainder having disappeared. When elaidin is fed as 40 per cent of the calories, absorption begins more promptly but continues more slowly than when elaidin alone is fed. The rate of absorption is constant. Owing to the preferential utilization of the protein and sugar in the diet by the body for fuel, a large percentage of the absorbed elaidic acid is deposited in the tissues.

Partition of the elaidic acid found in the body indicates the entrance of significant amounts of elaidic acid into the phospholipids of carcass and liver and the liver neutral fat. The chief site of deposition of elaidic acid is the adipose tissue, the elaidic acid content of which increases markedly as the absorption period lengthens.

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A BALANCE SHEET OF FAT ABSORPTION

II. THE STORAGE OF ELAIDIC ACID BY THE RAT OVER PERIODS OF SEVERAL DAYS

By MATHIAS F. F. KOHL

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

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In Paper I of this series (1) data were presented on the rates of absorption and storage of elaidic acid by young rats during a 24 hour period following the feeding of elaidin as the sole food and as 40 per cent of the calories. Since these experiments were only hourly studies continued through a period of 1 day following the feeding of the indicator fat, longer experiments were undertaken to study the absorption and storage of elaidic acid over periods of days.

EXPERIMENTAL

The procedures used in this investigation have already been described (1). In Experiment III of these studies young male rats weighing 100 ± 5 gm. were fasted 15 hours and then allowed to eat elaidin *ad libitum* as the sole food source throughout the various experimental periods, which ranged from 2 to 8 days. In Experiment IV Diet 262 (2) which contained elaidin as 40 per cent of its calories was fed *ad libitum* over periods of 2 to 5 days. Nearly all the animals used ate their diet regularly, as determined by repeated estimates of the food consumption. Those few not eating regularly were discarded. The fatty acids of the elaidin used had an iodine number 74. Consequently the value for elaidic acid represents only 82.2 per cent of the total fatty acid. The remaining saturated acids in the elaidin could not be traced after their absorption and so data on absorption and storage are expressed in terms of elaidic acid rather than the elaidin as a whole.

Fig. 1 represents the data for Experiment III on thirteen rats

which were fed elaidin as the sole food source. The partition between the various tissue lipids of the total elaidic acid found in the bodies of typical animals is presented in Fig. 2. Fig. 3 gives the summary for Experiment IV, in which thirteen animals were fed the Diet 262. The tissue balance sheets for typical animals chosen from Experiment IV are given in Fig. 4.

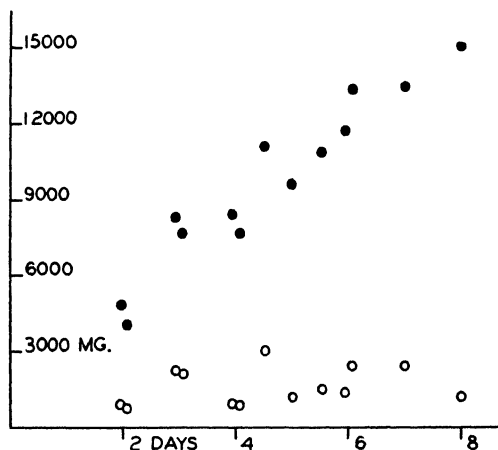


FIG. 1. Experiment III. Rats fed elaidin over a 2 to 8 day period. The elaidin fatty acids contained 82.2 per cent elaidic acid. Absorbed elaidic acid is indicated as ● and elaidic acid found in the tissues as ○. The unabsorbed elaidic acid in the gastrointestinal tract and feces amounted to 317 ± 107 mg. per animal. The feces practically disappeared during the period.

DISCUSSION

In order that these absorption experiments for long periods could be compared to those for short periods reported previously, the hourly absorption rate of elaidic acid was calculated for each animal from the data in Figs. 1 and 3. The average absorption rates of elaidic acid when calculated on an hourly basis for the thirteen rats receiving elaidin as their sole food and the other group of thirteen animals which were fed sugar and protein along with the elaidin were found to be 90 ± 10 mg. (range 112 to 78 mg.) and 59 ± 2 mg. (range 62 to 55 mg.) respectively. These values agree quite well with the hourly absorption rates of elaidic

acid during the initial 24 hour period of absorption. These values are 80 mg. when elaidin alone is fed, and 61 mg. when Diet 262 is given (1). Holmgren (3) has reported that some food is always present in the gastrointestinal canal of the rat. A maximum content occurs during the night, with a fall to a minimum during the day. Apparently the feeding habits of the rat and the slow rate

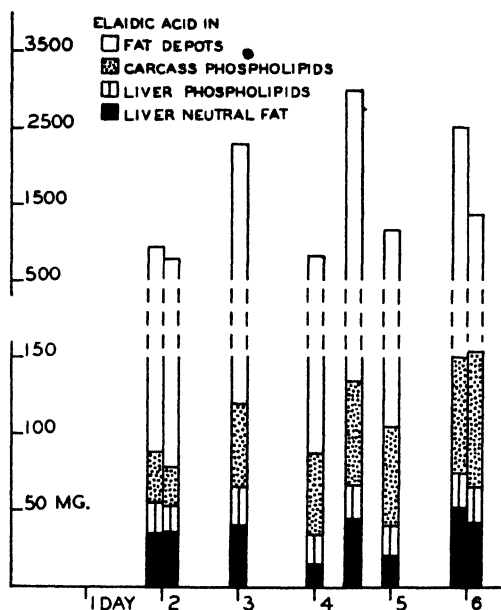


FIG. 2. The partition of the elaidic acid found in the bodies of typical animals chosen from Experiment III (Fig. 1). The total tissue elaidic acid of the animal is represented by the entire bar and the elaidic acid in the various tissue lipids by the designated bars that make up the whole.

at which elaidin is absorbed result in the maintenance of a fairly constant flow of this fatty acid through the intestinal wall.

The disappearance of elaidic acid in these experiments probably represents as closely the actual oxidation of the acid as can be determined at present. Transformation of elaidic acid to other acids by saturation or desaturation may take place but the measure of such a process, if occurring to any extent, has not been determined. The formation of oleic acid from elaidic acid by

steric rearrangement and the deposition in the tissues of an equilibrium mixture seem highly improbable.

The average hourly disappearance rate of elaidic acid can be calculated for each animal in Experiments III and IV from the difference between the elaidic acid absorbed and that found in the tissues at the end of the experimental period. In Experiment III the hourly disappearance rate of the absorbed elaidic acid is calculated to be 74 ± 4 mg. (range 65 to 84 mg.), while in Experiment IV the hourly utilization rate is 35 ± 2 mg. (range 32 to 38 mg.). These values are in excellent agreement with the rates

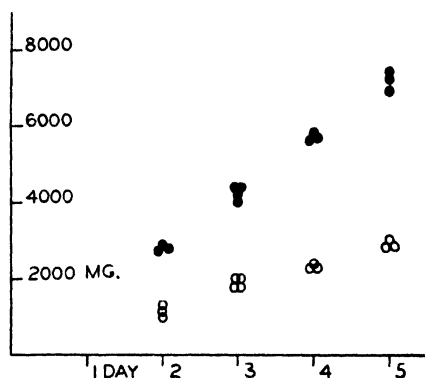


FIG. 3. Experiment IV. Rats fed Diet 262, containing elaidin as 40 per cent of the calories, over a 2 to 5 day interval. The elaidin fatty acids contained 82.2 per cent elaidic acid. Absorbed elaidic acid is indicated as ● and elaidic acid deposited in the tissues as ○. The unabsorbed elaidic acid in the gastrointestinal tract and feces amounted to 377 ± 171 mg. per animal.

obtained in the short experiments. During the initial 24 hours of absorption it was determined that absorbed elaidic acid disappears at the rate of 72 mg. per hour when elaidin is fed alone and at the rate of 35 mg. when fed with the sugar and protein (1).

The close agreement of the absorption and disappearance rates of elaidic acid in the experiments of short and long periods lends itself to the support of the conclusion drawn previously: that elaidic acid is absorbed by the rat at a fairly continuous and constant rate and is utilized or stored depending upon the immediate needs of the animal (1).

The animals in Experiment III lost considerable weight during the experimental period owing to diet deficiencies, while those in Experiment IV, receiving an essentially complete diet, gained weight. The final average weights of the two groups were 78 ± 4 and 104 ± 4 gm. respectively. In Experiment III despite the marked loss in body weight, the average fatty acid content of the depots increased to 3.80 ± 1.44 gm. compared to a control value

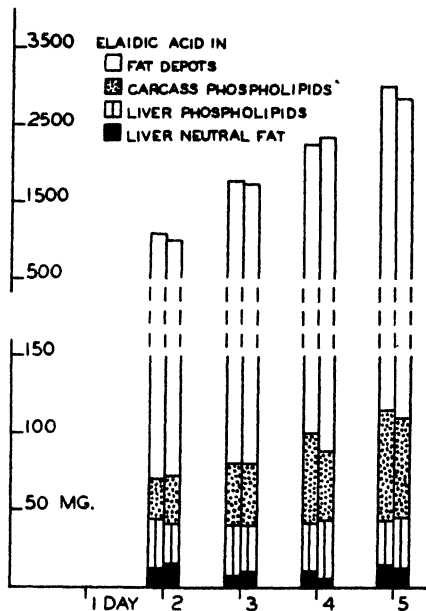


FIG. 4. The partition of the elaidic acid found in the bodies of typical animals chosen from Experiment IV (Fig. 3). The total tissue elaidic acid of the animal is represented by the entire bar and the elaidic acid in the various tissue lipids by the designated bars that make up the whole.

established in ten animals of 2.45 ± 0.73 gm. In Experiment IV the fatty acids in the adipose tissue increased to 5.80 ± 0.94 gm. A positive fat balance is indicated in both experiments.

The constancy of the disappearance rate from animal to animal in these experiments is quite striking. This is borne out in Experiment III, Fig. 1, where the absorption and deposition rates show considerable variation, while the rates of disappearance of

elaidic acid vary within narrower limits. Therefore the amount of fat stored in the tissues of any one animal seems directly determined by the magnitude of the absorption rate—the greater the rate of absorption, the more fat absorbed in excess of metabolic needs and consequently the more fat that has to be stored.

The question may be raised as to the possibility that a cycle occurs in the depots with periods of deposition of the excess calories absorbed alternating with periods of withdrawal of fat later when absorption does not supply the metabolic needs, and that the rates obtained for disappearance and storage of elaidic acid in the experiments reported here are only the mean of such a cycle. The data of this study do not indicate that cyclic deposition and withdrawal of elaidic acid occur to any great extent in the rat adipose tissue. The slow withdrawal of elaidin from the depots after its deposition further substantiates this conclusion (unpublished data). It must not be inferred, however, that withdrawal of elaidic acid from the depots does not occur during the period when elaidin is being absorbed. Since the values for the hourly absorption and disappearance rates of elaidic acid are only averages, fluctuations must be expected from hour to hour owing to periods of rest, sleep, activity, etc. Consequently it is not unlikely that during a period of maximum activity on the part of the animal and minimum absorption some demand must be made on the depots to offset the energy deficit. This is indeed a function of the fat reserve, to act as an energy buffer in case such a condition arises. The fact that it can be shown that elaidic acid does disappear slowly from the depots when the animal is eating an elaidin-free diet requires some such explanation.

In Experiment III (Fig. 2) the amount of elaidic acid appearing in the liver neutral fat is quite large and makes up 35 ± 5 per cent of the fatty acids in this fraction. The accumulation of neutral fat fatty acid during the dietary régime is quite large, amounting to 104 ± 30 mg. in spite of a marked reduction in the liver weight to 3.84 ± 0.27 gm. The livers of ten control animals weighed 5.18 ± 0.75 gm. and contained 29 ± 5 mg. of neutral fat fatty acid. The accumulation of fat in the liver on a high fat diet is not unusual. However, elaidin accounts for only part of the excess fat in these livers. The uptake of elaidic acid by the phospholipids of the liver is essentially complete by the 2nd day, after which the elaidic acid makes up 21 ± 3 per cent of the 94 ± 6

mg. of fatty acid. On the other hand, the entrance of elaidic acid into the phospholipids of the carcass continues slowly throughout the period. Elaidic acid makes up about 5 per cent of the fatty acids after the 2nd day of feeding and increases to 12 per cent by the 6th day. These findings in respect to the entrance of elaidic acid into phospholipids of liver and carcass are essentially the same as Sinclair's (4). The greatest percentage of elaidic acid is present in the adipose tissue. Variations in the amounts deposited at the various intervals are the result of differences in the ingestion of the fat by the various animals.

In the balance sheet of tissue elaidic acid for Experiment IV (Fig. 4) it is again seen that the increase in body elaidic acid as the absorption period lengthens is primarily reflected in the depot fat. Elaidic acid is found in small amounts in the liver neutral fat fatty acids. This represents a small fat store in these animals, amounting to 38 ± 7 mg. with an elaidic acid content of 27 ± 3 per cent. The elaidic acid concentration in the liver phospholipids has reached a maximum by the 2nd day and remains quite constant thereafter. The 140 ± 12 mg. of liver phospholipid fatty acid contain 23 ± 3 per cent elaidic acid. The liver weight is 6.60 ± 0.57 gm. The entrance of elaidic acid into the carcass phospholipids continues slowly throughout the period studied. After 2 days of feeding Diet 262 the elaidic acid content of the carcass phospholipid fatty acids is approximately 4 per cent and increases to 9 per cent by the 5th day.

In comparing the tissue balance sheets¹ of Experiments III and IV a number of interesting points are brought out. In Experiment III, despite the fact that the liver weight is considerably lower than in Experiment IV, the amount of elaidic acid in the liver neutral fat fraction is considerably greater. This no doubt is the result of feeding elaidin as the sole diet. Differences in the elaidic acid content of the liver phospholipids are quite striking. This is not surprising in view of the marked differences in the liver weights. In Experiment III the average liver weight is

¹ These balance sheets are incomplete in regard to the elaidic acid in the gastrointestinal tissue. The amount present, however, would not be sufficiently great to cause any appreciable change in the value of the tissue elaidic acid, since the elaidic acid found in tissue other than the depot fat is quite small.

3.84 gm. with a phospholipid fatty acid content of 94 mg.; in Experiment IV the liver weighs 6.60 gm. and contains 140 mg. of phospholipid fatty acid. The percentage of elaidic acid in the liver phospholipid fatty acids is the same in the two experiments, about 22 per cent. If the elaidic acid-containing phospholipid in the liver is a metabolic phospholipid synthesized in this organ and then transported to the tissues for catabolism, the rate of turnover of this lipid in the liver in Experiment III is not only enhanced by the high utilization of fat, but also by the decrease in size of the organ itself. In comparing the amounts of elaidic acid in the phospholipids of the carcass, cognizance should be taken of the loss in body weight when elaidin alone is fed and the maintenance or increase in weight on Diet 262. The carcass phospholipid fatty acid content per 100 gm. of tissue is 0.85 gm. in both experiments. In Experiment III the average carcass phospholipid fatty acid content is 0.56 gm. per animal (average carcass weight 66 gm.) as compared to 0.71 gm. for Experiment IV (average carcass weight 84 gm.). Yet the absolute amounts of elaidic acid entering the phospholipids are nearly the same in the two experiments. This, of course, means that the replacement of the carcass phospholipid fatty acids by elaidic acid goes on more rapidly in Experiment III, in which elaidin alone is fed. The difference in the adipose tissue elaidic acid in the two experiments has been discussed.

SUMMARY

Further study on the absorption and storage of elaidic acid by the rat, when elaidin is fed alone and when fed with sugar and protein, over periods of days lends itself to the support of the conclusion that the indicator fat is absorbed at a fairly continuous and constant rate and is utilized or stored depending upon the immediate needs of the body. When elaidin alone is fed, only a small part of the absorbed elaidic acid finds its way into the depots; when fed along with protein and sugar, a large part escapes utilization and is deposited.

Partition of the elaidic acid in the tissues indicates the rapid entrance of elaidic acid into the liver phospholipids and a slow entrance into those of the carcass. Elaidic acid is present also in the liver neutral fat. The chief site of the deposition of the

elaidic acid is the adipose tissue, the elaidic acid content of which increases markedly during the course of the experimental period.

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A BALANCE SHEET OF FAT ABSORPTION

III. THE DISAPPEARANCE OF ELAIDIC ACID FROM THE TISSUES OF THE RAT

By MATHIAS F. F. KOHL

*(From the Department of Biochemistry and Pharmacology, School of Medicine
and Dentistry, The University of Rochester, Rochester, New York)*

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In connection with the problem of the storage and withdrawal of fat from the depots, the work of Gage and Fish (1) is a classic. These investigators fed young rats bread that was spread with butter stained with Sudan III. Prompt entrance of the diet fat into the depots several hours after feeding was indicated by a pinkish coloration of the depots. The intensity of the color increased as absorption continued. 20 days were required for the color produced by this feeding to disappear from the adipose tissues. The inertness of fat once it has been deposited in the depots is also indicated by the findings of Ellis and Isbell (2) on the pig, and of Brown and Rawlins (3) and Anderson and Mendel (4) on the rat. On the other hand a more rapid turnover of depot fat is reported by Schoenheimer and Rittenberg to take place in the mouse (5).

In Experiment V of the studies reported here an attempt was made to study as quantitatively as possible the disappearance of elaidic acid from the depots after its deposition. To what extent the disappearance of a "tagged" fat in the body once it has been deposited can be attributed to processes other than combustion cannot at present be stated. Consequently it is assumed that disappearance of elaidic acid indicates its withdrawal from the depots in a mixture with other acids for utilization.

EXPERIMENTAL

The experimental methods have been reported in Paper I (6). Young male rats weighing 100 ± 5 gm. were fasted 15 hours and then fed Diet 262 (7), containing elaidin as 40 per cent of the

calories, for 72 hours. The elaidin fatty acids had an iodine number 74 and contained 82.2 per cent elaidic acid. The animals were allowed to eat *ad libitum* and observations were made to insure prompt, ample, and regular eating of the food. The few animals failing to do so were discarded. After 3 days on Diet 262 the rats were shifted back to the calf meal (8) which was continued until the time of sacrifice. During the period of the calf meal feeding records of the food intake were made every 2 days. Without exception the animals showed an increase in food consumption paralleling gain in weight.

To correct for any possible loss of elaidic acid in the feces, the feces passed during the 3 day period on Diet 262 were collected and analyzed. Twenty-two animals excreted a total of 27 gm. of dried feces during the 3 day period. Analysis indicated the presence of 19 mg. of elaidic acid per 1 gm. of feces. This indicates that the loss of elaidic acid in the feces is negligible, which confirms similar findings by Barbour (9) and Sinclair (personal communication).

In Experiment IV of these studies (Paper II (10)) it was found that when elaidin containing 82.2 per cent elaidic acid is fed as 40 per cent of the calories in Diet 262 over a 2 to 5 day period the average hourly rates of absorption and disappearance of elaidic acid are quite constant from animal to animal. These constants are 59 and 35 mg. respectively. In calculating the data in Experiment V, the amount of elaidic acid consumed in the food during the 3 day period was divided by the average hourly absorption rate of 59 mg. The value obtained is the corrected absorption time for the ingested elaidic acid. This calculation is necessary, since the amount of unabsorbed diet in the gastrointestinal tract at the end of the 72nd hour, when diets were changed, cannot be estimated. The corrected value for the period during which elaidic acid is being absorbed then makes possible the calculation of the amount of absorbed elaidic acid disappearing. The estimate for the amount of elaidic acid stored in the body during the 3 days of Diet 262 can then be compared with the amount of elaidic acid remaining in the tissues 5 to 30 days after the calf meal diet has been resumed. These data for Experiment V are given in Fig. 1.

DISCUSSION

About 30 days are required to clear the depots of elaidic acid that was deposited during the 3 days of feeding elaidin as 40 per cent of the calories. The disappearance of elaidic acid seems to occur a little more rapidly in the early part of the experiment. This may be partly explained on the basis of a higher percentage of elaidic acid in the depots at the beginning of the calf meal

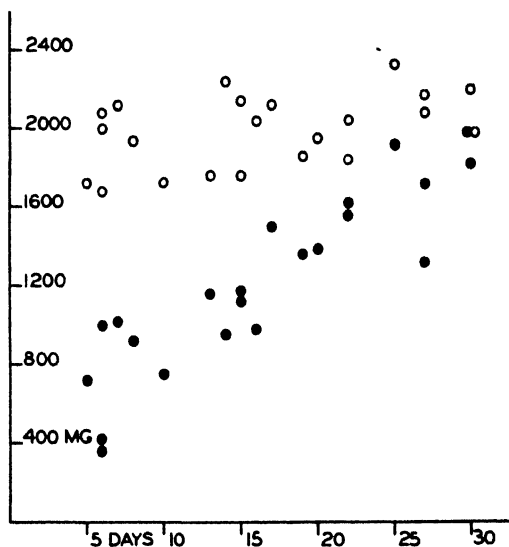


FIG. 1. Experiment V. The disappearance of elaidic acid from the body of the rat. The elaidic acid deposited in the tissues during the 3 day period of feeding Diet 262 is indicated as O and the elaidic acid having disappeared during the interval of calf meal feeding as ●. The difference represents the elaidic acid found in the body of the rat.

feeding period than later when the percentage has been lowered not only by the withdrawal of elaidic acid but also a subsequent deposition of fat from the calf meal. Consequently the percentage of elaidic acid withdrawn in a mixture of fat from the depots becomes less and less as the calf meal feeding period lengthens. The data indicate the inertness of fat once it has been deposited in the depots of the rat.

The partition of the tissue elaidic acid of animals in Experiment V indicates that all but a few mg. of the elaidic acid are present in the adipose tissue. The liver neutral fat contains on the order of 1 mg. of elaidic acid throughout the period. Several mg. are present in the liver phospholipid fatty acids a week after resumption of the calf meal but after that the amount becomes negligible. In the carcass phospholipids, in which the rate of turnover of elaidic acid is quite slow, 30 mg. are found 1 week after calf meal refeeding. The amount falls off gradually as the period is lengthened.

Sinclair has reported (11) that the rate of entrance of elaidic acid into, and disappearance from the phospholipids is rapid in the liver, and comparatively slow in muscle. The process is essentially complete in the liver within 1 day, but in muscle only after a period of many days. On the basis of this evidence and former evidence on the selective retention by the liver of highly unsaturated acids in the diet (12), Sinclair concludes that two types of phospholipid are present in the liver tissue: the more highly unsaturated ones that function in the essential make-up of the cell, and the less unsaturated, represented by those containing elaidic acid, which function as intermediary products of fat metabolism. The phospholipids of muscle, on the other hand, are believed to be mainly of the non-metabolic type.

Figs. 2 and 3 present data on the turnover of elaidic acid in the phospholipids of the rat carcass and liver obtained in this and previous experiments. The carcass phospholipids are made up primarily of those in the skeletal muscle. The entrance of elaidic acid into the phospholipids was determined in Experiment II (6) and Experiment IV (10) reported previously in which young male rats were fed Diet 262 for periods of 8 hours to 5 days. The disappearance of elaidic acid from phospholipid was determined in Experiment V of this paper, in which, after eating Diet 262 for 3 days, the animals were put back on the stock diet. The amount of elaidic acid in the phospholipids is indicated by the increase in the iodine number of the solid fatty acids isolated over the control iodine numbers that have been previously determined for the naturally occurring solid acids of the rat lipids (6).

The findings of this study are in agreement with those of Sinclair. The entrance of elaidic acid into carcass phospholipid is slow compared to the rapid rate in the liver. The rate of dis-

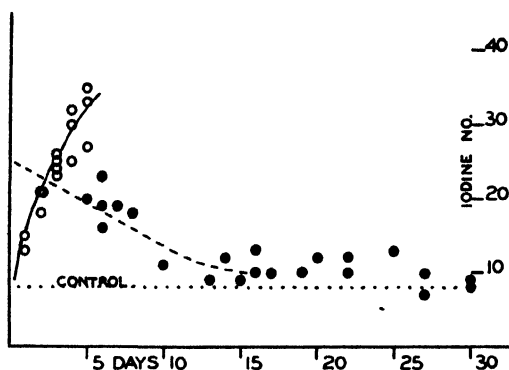


FIG. 2. The turnover of elaidic acid in the carcass phospholipids of the rat. The entrance of elaidic acid into the phospholipids during Diet 262 is indicated as ○. The elaidic acid remaining in the phospholipids after the various intervals of calf meal feeding, following the 3 day period of Diet 262, is given as ●. The iodine number of the phospholipid solid acids is used as an index of the amount of elaidic acid present.

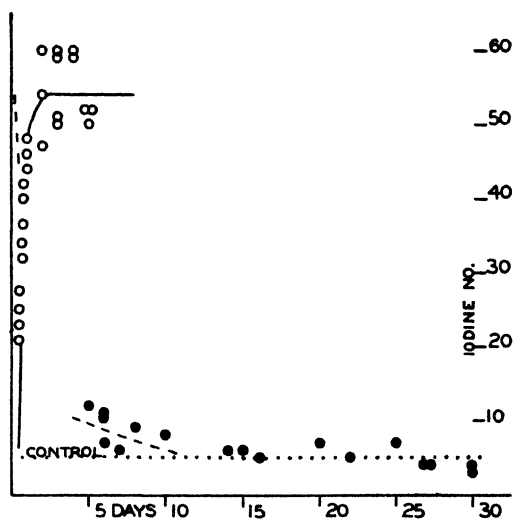


FIG. 3. The turnover of elaidic acid in the liver phospholipids of the rat. The entrance of elaidic acid into the phospholipids during Diet 262 is indicated as ○. The elaidic acid remaining in the phospholipids after the various intervals of calf meal feeding, following the 3 day period of Diet 262, is given as ●. The iodine number of the phospholipid solid acids is used as an index of the amount of elaidic acid present.

appearance of elaidic acid from the carcass phospholipids is quite slow, the iodine numbers of the solid acids falling slowly until the 10th day and then leveling off. The iodine numbers, however, continue to remain significantly above the control level, even after 3 weeks. In the liver phospholipids the persistence of small amounts of elaidic acid is also found after the immediate precipitous fall toward normal. It cannot be said at present whether this persistence of small amounts of elaidic acid in the phospholipids of the carcass and liver is due to a retention of elaidic acid-containing phospholipids by these tissues or due to a new formation from elaidic acid subsequently coming out of the depots.

SUMMARY

Elaidic acid when once deposited in the fat depots of the rat disappears at a slow rate, indicating that the depot fat in the rat is relatively inert. Over 30 days are required to clear the reserves of elaidic acid that is deposited during a 3 day period of feeding elaidin as 40 per cent of the caloric intake.

The findings of Sinclair on the slow rate of turnover of elaidic acid in the muscle phospholipid of the rat and the rapid rate in liver phospholipid have been confirmed.

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ISOEQUILIN A

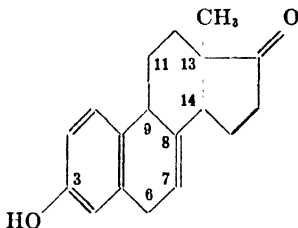
By H. HIRSCHMANN* AND OSKAR WINTERSTEINER

(From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York)

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In the course of an investigation of the estrogenic diols present in the urine of pregnant mares (1) a substance was isolated which seemed to differ from estradiol by the presence of a fourth double bond in conjugation with the aromatic ring. As we were unable to secure sufficient amounts of this compound to elucidate its structure, we have endeavored to prepare substances containing such an arrangement of double bonds from other more readily available estrogens.

Equilin (1) appeared to be particularly suitable for this purpose, as it already contains four ring double bonds. It should be com-



paratively easy to shift the double bond between C₇ and C₈ into a position conjugated with Ring A; that is, either between C₆ and C₇, or C₈ and C₉, or C₉ and C₁₁. Treatment at elevated temperature with sodium ethylate or amylate, while effective in rearranging 1,4-dihydronaphthalene into 1,2-dihydronaphthalene (2), failed to bring about such a transformation with equilin. Neither did the customary procedure of isomerizing unsaturated steroids

* Columbia University Fellow, 1937-38.

by treating a chloroform solution with dry hydrogen chloride effect such a change. Shifting of the double bond occurred, however, when equilin was heated in a mixture of concentrated hydrochloric and acetic acids. That the double bond had migrated into a position conjugated with Ring A was indicated by the absorption spectrum of the crude reaction product, which exhibited a very high maximum between 264 and 272 $m\mu$ and thus markedly differed from that of equilin (Fig. 1, Curve I). On fractional recrystallization this material yielded apparently

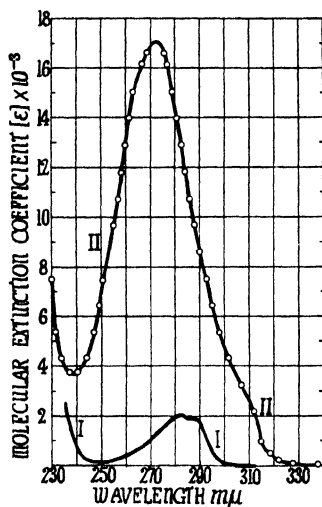


FIG. 1. Absorption spectra of equilin (Curve I) and Isoequilin A (Curve II) (alcohol).

homogeneous platelets (Fig. 2) melting at 231°. Whereas the final preparations from three different runs appeared identical as judged by crystal form, melting point, and absorption spectrum (Fig. 1), two of them showed approximately the same rotatory power ($[\alpha]_D = +222^\circ$ and $+224^\circ$ respectively), while the specific rotation of the third was appreciably higher ($[\alpha]_D = +241^\circ$). Neither fractional crystallization nor adsorption on alumina was of much avail in fractionating the products with either the higher or the lower rotation. By chromatographic analysis there could be removed in each instance a small crystalline fraction which had

a rotation considerably lower than the bulk of the material. While these findings leave some doubt as to the absolute homogeneity of these preparations, we have nevertheless attempted to determine the structure of the material exhibiting a specific rotation of $+222^\circ$.

The substance, which will be referred to as Isoequilin A, corresponds in composition and in the function of its oxygen atoms with equilin. An acetate and a semicarbazone have been prepared. The absorption spectrum (Fig. 1, Curve II) clearly indicates that the product contains a double bond in conjugation with Ring A. In order to differentiate between the three possible locations of

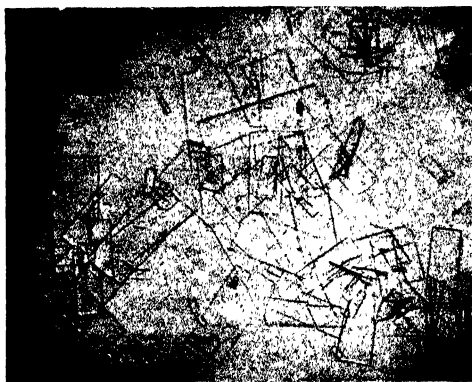


FIG. 2. Crystals of Isoequilin A (from 80 per cent ethyl alcohol)

this double bond we followed a procedure employed by Serini and Logemann (3) to secure additional evidence for the position of the fourth double bond of equilin. These authors prepared an equilin-*cis*-glycol by treatment with osmium tetroxide according to the method of Criegee (4) and determined in this compound the number of hydroxyl groups resistant to acetylation. We were able to prepare equilin glycol¹ in good yield from equilin acetate; when, however, the acetate of Isoequilin A was subjected to the same procedure, a crystalline substance melting at 204° was obtained, which gave results agreeing with $C_{18}H_{20}O_3$, in-

¹ Our preparation melted at 254° (corrected); the melting point given by the German authors is 245° (uncorrected?).

dicating that only 1 atom of oxygen had entered the molecule. The absorption spectrum (Fig. 3) closely resembled that of Isoequilin A; this new product therefore also contains a double bond in conjugation with Ring A. The most plausible explanation for the formation of such a compound is that the osmic ester of the glycol normally formed as the primary reaction product broke down with the formation of a double bond. Our failure to isolate a glycol indicates that the fourth double bond in Isoequilin A is not located between C₆ and C₇, for a glycol with two secondary hy-

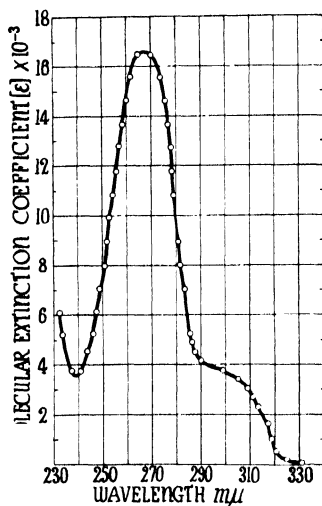
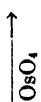
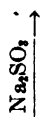
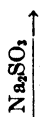
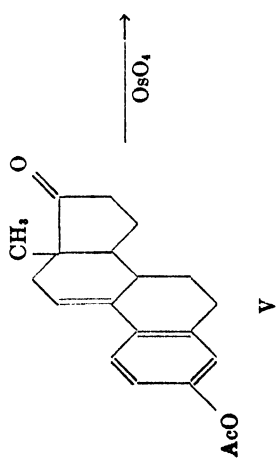
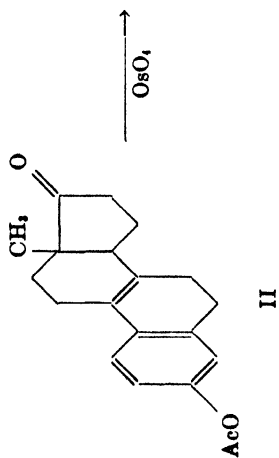
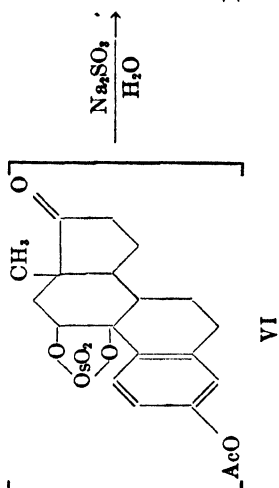
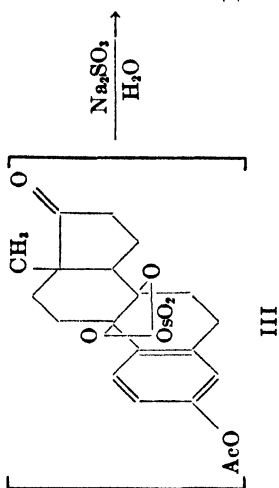
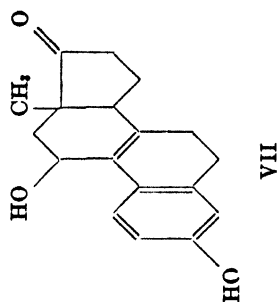
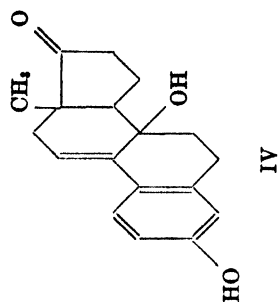


FIG. 3. Absorption spectrum of 14-epi- Δ_9 -11,8-hydroxyequilin

droxyl groups, such as would be expected from a Δ_{6-7} -equilin, should not tend to split out water so readily. Furthermore, of compounds which absorb more intensely than estrone, the only one which could have arisen from a 6,7-glycol by the removal of 1 mole of water is 6-keto-estrone. The absorption spectrum of 6-keto- α -estradiol² has been measured and found to be entirely different from that of the substance melting at 204° (maxima at 257 mμ and 333 mμ, minima at 241 and 285 mμ). As there is no other position in the molecule in which a keto group could appre-

² Wintersteiner, O., unpublished experiments.



ciably influence the absorption spectrum of the phenolic ring, the analytical figures in conjunction with the spectrographic findings lead to the conclusion that the 3rd oxygen atom must be present as a hydroxyl group. It is possible to formulate the reaction in accordance with the observations if the starting material has the structure of an acetate of either Δ_{8-9} -equilin (II) or Δ_{9-11} -equilin (V).

The substance melting at 204° yielded a monoacetate when treated with acetic anhydride and pyridine at room temperature. This indicates that the newly formed hydroxyl group is tertiary, as in Formula IV. It might be argued that a secondary hydroxyl group at C_{11} , such as is present in the alternative Formula VII, would likewise be resistant to acetylation. However, those structural features which are held responsible for the chemical inertness of an 11-hydroxyl group, as in the case of the adrenal compounds (5), are absent from our compound. The main factor which could confer steric hindrance on an oxygen atom in position 11, namely the methyl group on C_{10} , is lacking in the estrogens. Furthermore, the presence of a double bond between C_8 and C_9 abolishes the *trans* relation of Rings B and C, which could conceivably lead to interference if the 11-hydroxyl lay on the same side of Ring C as carbon atoms 10 and 1. For these reasons we assign Structure IV, and not VII, to the substance melting at 204° , and consequently Isoequilin A is considered to be Δ_{8-9} -equilin. This assignment of structure is valid only if it be assumed that no allylic rearrangement has taken place during any of the reactions.

A somewhat analogous observation has been made by Windaus, Linsert, and Eckhardt (6). When the acetate of $\Delta_{6-7, 8-9}$ -cholestadienol-3 (isodehydrocholesterol acetate) was treated with perbenzoic acid, 9-hydroxy- $\Delta_{6-7, 8-14}$ -cholestadienol-3 acetate was formed instead of an 8,9-oxide or an 8,9-*trans*-glycol. Even more striking is the behavior of the acetate of δ -cholestenol, which is believed to be a Δ_{8-9} -cholestenol, towards the same reagent. Again an α, β -unsaturated tertiary alcohol, Δ_7 -cholesten-3,9-diol, is formed.

In order to secure additional evidence for the proposed structure, Isoequilin A was treated with palladium at 80° . Equilin is transformed by this treatment into the naphtholic compound

equilenin (7). A Δ_{8-9} -equilin should also be readily dehydrogenated under these conditions (8), whereas no such reaction is to be expected from a Δ_{9-11} -equilin unless it should first rearrange to a product containing the fourth double bond in Ring B. A substance with the absorption spectrum of equilenin was actually formed, in a yield of about 75 per cent. The product, which was purified by means of its picrate, melted at 262° . The analytical results corresponded to the formula $C_{18}H_{18}O_2$, which is the composition of equilenin. However, the dextrorotation of the compound was 70° higher than that of equilenin. Also, when mixed with equilenin (m.p. 258°), the substance showed a distinct depression of its melting point. The substance is therefore not equilenin but one of its stereoisomers. There are only two centers of asymmetry in the molecule; namely, C_{13} and C_{14} . Epimerization at C_{13} could hardly have occurred, but it is easy to picture a reversal of the configuration of C_{14} by assuming that the rearrangement of equilin to Isoequilin A proceeded with the intermediate formation of a Δ_{8-14} -equilin. It could alternatively be assumed that the epimerization occurred during the catalytic dehydrogenation of Δ_{8-9} -equilin to the equilenin isomer, but it is difficult to see why this should not also take place when equilin is subjected to the same process. We therefore believe that Isoequilin A differs from equilin not only in the position of the double bond, but also in the configuration of C_{14} . According to this view, Isoequilin A is really a 14-epi- Δ_{8-9} -equilin. The fact that different preparations of Isoequilin A showed discrepancies in their rotatory power may be ascribed to the varying extent to which epimerization took place during the acid treatment and to the difficulty of completely separating the epimers.

The absorption spectrum of Isoequilin A differs sufficiently from that of the diol isolated from urine to justify the conclusion that the fourth double bond occupies different positions in the two compounds. We provisionally suggest that the urinary substance has the structure of a Δ_{6-7-17} -dihydroequilin.

Furthermore, it can be stated that the Δ_{8-9} -equilin described in this paper is different from the two isomers of equilin described in the literature; namely, the isoequilin of Inhoffen (9), and the hippulin of Girard and coworkers (10).

EXPERIMENTAL

Isoequilin A—To a mixture of 135 cc. of glacial acetic acid and 90 cc. of concentrated hydrochloric acid were added 989 mg. of equilin ($[\alpha]_D^{21} = +317^\circ$ in absolute alcohol; absorption spectrum as given in Fig. 1). The solution was heated under a reflux in an atmosphere of CO_2 for 80 minutes. A strong green fluorescence developed but almost disappeared towards the end of the heating period. The pink solution was chilled and distributed between 600 cc. of water and 400 cc. of benzene. The yellow benzene layer was washed four times with 300 cc. of water, the benzene was distilled off *in vacuo*, and the residue recrystallized twice from a mixture of benzene and acetone (4:1). The material obtained by evaporating the mother liquors (446 mg.) oxidized in contact with air, with the formation of colored products, and was set aside. The crystals (536 mg.) were dissolved in a mixture of 150 cc. of benzene and 15 cc. of acetone, and the solution was filtered through a 12 cm. column of Brockmann's aluminum oxide (inside diameter 2 cm.). The chromatogram was developed by using 65 cc. of the same mixture of solvents. The filtrate contained 20 mg. of colorless crystals ($[\alpha]_D^{27} = +177^\circ$ in alcohol; the absorption spectrum showed a maximum at $273 \text{ m}\mu$ ($\epsilon = 14,000$), and a minimum at $240 \text{ m}\mu$ ($\epsilon = 3500$)). The top 5 mm. of the column retained most of the pigment and were discarded. The succeeding 75 mm. and the remaining 40 mm. of the column were immediately eluted with acetone and yielded 250 mg. ($[\alpha]_D^{25} = +218^\circ$) and 217 mg. ($[\alpha]_D^{23} = +215^\circ$) of crystals respectively.³ The two fractions, combined and recrystallized twice from 80 per cent ethyl alcohol, yielded 320 mg. of thin rectangular plates which melted⁴ at 231° after incipient decomposition at 227° . $[\alpha]_D^{25} = +222^\circ$ (0.5 per cent in ethanol).

Analysis— $\text{C}_{13}\text{H}_{20}\text{O}_2$. Calculated. C 80.55, H 7.52
Found. " 80.50, " 7.61

The product exhibited green fluorescence when dissolved in concentrated sulfuric acid. It coupled readily with an acid

³ Though the alcoholic solutions of these fractions were clear and almost colorless, they did not transmit sufficient light either at $589 \text{ m}\mu$ or at $546 \text{ m}\mu$ for an accurate determination of their rotatory power. The disturbing substance could be eliminated by one recrystallization.

⁴ All melting points given are corrected.

solution of *p*-nitrodiazobenzene, yielding a brown-orange pigment; in this respect it resembles the diol isolated from mare urine. Compound formation with picric acid or with quinoline could not be observed.

According to the report of Dr. Charles Mazer (of Philadelphia) the compound possesses one-fifth of the potency of estrone when tested on the ovariectomized rat. The assay technique has been given in a previous paper (11).

Semicarbazone of Isoequilin A—A solution of 21.5 mg. of Isoequilin A and 150 mg. of semicarbazide acetate in 4.5 cc. of 90 per cent ethanol was refluxed for 2 hours on a steam bath. After the evaporation of some of the alcohol a small volume of water was added. The gelatinous product was washed with 50 per cent alcohol and thoroughly with water (yield 20 mg.). Small crystals were obtained by recrystallization from ethanol. The semicarbazone turned brown at about 180° and gradually decomposed when the temperature was raised to 230°. The analytical sample was dried for 2 hours at 110° and 6 mm. pressure.

<i>Analysis</i> — $C_{19}H_{23}N_3O_3$.	Calculated.	C 70.11, H 7.13, N 12.93
$C_{19}H_{23}N_3O_3 \cdot \frac{1}{2}H_2O$.*	"	" 68.22, " 7.24, " 12.57
	Found.	" 68.37, " 7.27, " 12.88
	"	" 68.42, " 7.41

Acetate of Isoequilin A—217 mg. of Isoequilin A were dissolved in 6 cc. of pyridine and 3 cc. of acetic anhydride. The mixture, which turned pink on standing, was kept at 21° for 12 hours and was then chilled. 1.5 cc. of ice-cold water were added. The product was taken up in ether and repeatedly washed with hydrochloric acid, sodium carbonate solution, and water. The ether yielded on evaporation 245 mg. of a red oil that crystallized on standing. The red pigment which was carried along on recrystallization could be removed by treating a dry ethereal solution with charcoal, or by distillation in a high vacuum. The melting point of a preparation that had been purified by both these methods and repeatedly recrystallized from dilute alcohol (rectangular prisms), although not sharp, was the same as that of

* Difficulties were also encountered by Butenandt *et al.* (12) in removing the water of crystallization from estrone semicarbazone.

less thoroughly purified material (softening at 83°; clear melt at 95°).

Analysis—(Sample dried at 56°, 6 mm. Hg)

$C_{20}H_{22}O_3$. Calculated. C 77.38, H 7.15

Found. " 77.41, " 7.13

The absorption spectrum of the acetate (hexane) showed a maximum at 268 $m\mu$ and a minimum at 238 $m\mu$.

14-Epi- Δ_9 -11-8-Hydroxyequilin—68.6 mg. of the acetate of Isoequilin A were dissolved in 3 cc. of freshly distilled, dry ether. 63.5 mg. of osmium tetroxide were added. After 5 minutes a brown precipitate appeared in the solution. The mixture was allowed to stand for 42 hours. The precipitate (100 mg.) was centrifuged off, washed twice with 0.5 cc. of dry ether, and treated with 0.86 gm. of crystalline sodium sulfite and 25 cc. of 20 per cent alcohol. The suspension was refluxed for 2 hours. The black precipitate which had formed on heating was filtered off and thoroughly washed with hot 95 per cent and 50 per cent alcohol. The filtrate and the washings were combined, concentrated to about 10 cc., and kept in the ice box overnight. A crystalline precipitate was obtained which was centrifuged off and washed with water (Fraction A; 9 mg.). Mother liquor and washings were extracted with 36 volumes of highly purified chloroform in eight portions. The chloroform solution, dried with sodium sulfate and evaporated, yielded 12 mg. of a colorless oil which on standing oxidized with the formation of brown pigments. This fraction was therefore discarded. The original ether supernatant and washings were evaporated (27 mg.) and worked up in the same way as the precipitate that had been removed by centrifugation. Only 4.8 mg. of crystalline material could eventually be recovered from this fraction. Neither could the yield be materially increased by extraction of the various aqueous solutions with chloroform after acidification, nor by extracting the osmium precipitate in a Soxhlet apparatus with alcohol. The saponification of the osmiate must have been complete, because the aqueous phase, when treated again with sodium sulfite, remained clear. The yield of crystalline material was about 25 per cent.

Fraction A was recrystallized twice from a mixture of benzene and acetone. Colorless platelets were obtained which melted

at 204° with decomposition. Two different runs yielded an identical product.

Analysis— $C_{18}H_{20}O_3$. Calculated. C 76.02, H 7.09
Found. " 75.85, " 7.30

The compound yields an orange-colored pigment on coupling with an acid solution of *p*-nitrodiazobenzene.

14-Epi- Δ_9 -11-8-Hydroxyequilin-3 Acetate—11.9 mg. of the hydroxyequilin were dissolved in 0.8 cc. of dry pyridine and 0.4 cc. of acetic anhydride. The mixture was kept at room temperature for 12 hours and then worked up in the same manner as the acetate of Isoequilin A. 13.5 mg. of a colorless oil were obtained which defied all efforts to crystallize it. It was analyzed after drying to constant weight.

Analysis— $C_{20}H_{22}O_4$. Calculated. C 73.58, H 6.80
Found. " 73.62, " 6.86

Dehydrogenation of Isoequilin A—A round bottom flask containing 50 mg. of Isoequilin A, 315 mg. of palladium black, prepared according to Tausz and von Putnoký (13), and 25 cc. of absolute alcohol was evacuated and sealed. The flask was shaken for 16 hours at 80°. The catalyst was filtered off and the colorless filtrate was evaporated in a current of carbon dioxide. The residue was dissolved in warm absolute alcohol containing 58 mg. of picric acid. The light orange picrate formed was recrystallized from a 5 per cent solution of picric acid in ethanol, and then decomposed in the usual manner, giving 23 mg. of colorless needles which after recrystallization from dilute alcohol melted at 262°. As in the case of equilenin the melting is preceded by the formation of a red pigment a few degrees below the melting point. A mixture with an authentic sample of equilenin melting at 259° started to turn red at 227° and melted at 251.5°. $[\alpha]_D^{30} = +160^\circ$ (0.4 per cent in ethanol). The absorption spectrum agreed with that of equilenin within the limit of the method.

Analysis— $C_{18}H_{18}O_2$. Calculated. C 81.16, H 6.82
Found. " 80.97, " 6.67

SUMMARY

Equilin is converted by treatment with acid into a double bond isomer, Isoequilin A, to which the structure of a 14-*epi- Δ_9 -9-*

equilin has been assigned. The new compound yields on catalytic dehydrogenation a stereoisomer of equilenin, 14-epi-equilenin, which differs from equilenin by the configuration of carbon atom 14. Isoequilin A reacts with osmium tetroxide with the formation of a hydroxyequilin, probably 14-epi- Δ_{9-11} -8-hydroxyequilin. Spectroscopic measurements indicate that the fourth double bond in Isoequilin A does not occupy the same position as that in the diol recently isolated from the urine of pregnant mares. Isoequilin A possesses about one-fifth of the estrogenic potency of estrone.

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CHANGES OF ORGANIC ACID-SOLUBLE PHOSPHORUS, DIPHOSPHOGLYCERATE, ADENOSINETRIPHOSPHATE, AND INORGANIC PHOSPHORUS IN THE BLOOD CELLS OF RATS DURING THE DEVELOPMENT AND HEALING OF RICKETS

By S. RAPOPORT AND GEORGE MARTIN GUEST

(From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati)

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Changes of organic phosphorus in the blood in rickets were first reported in 1920 by Iverson and Lenstrup (1). In rachitic infants they found that during the healing period the total acid-soluble P in the whole blood increased from 20 to 27 mg. per 100 cc.; no data on the volume of cells in their blood samples were reported. Zucker and Gutman (2) found no reduction of organic acid-soluble P in the whole blood of rachitic infants or rachitic rats, compared with values determined in non-rachitic individuals. Robison and Soames (3) reported that in the whole blood of rachitic rats fed the McCollum diet, No. 3143 (4), the concentration of phosphoric esters hydrolyzable by bone enzyme was below normal; after the administration of cod liver oil to these rats, while the level of inorganic P in the blood rose nearly to normal, the amounts of P esters were not significantly increased. György (5) found no change in the organic acid-soluble P in the whole blood of rachitic infants during the period of healing. Wilder (6) observed increases of organic acid-soluble P in the whole blood of rachitic rats subjected to fasting, a procedure which induces healing, as had been previously described by McCollum *et al.* (7) and by Cavins (8). Sokolovitch (9) failed to find changes of organic P in the blood cells of one rachitic infant. Bakwin, Bodansky, and Turner (10) found the readily acid-hydrolyzable P reduced in the blood cells of rachitic infants, and similar findings in the blood of rachitic rats were reported by Jacobsen (11). Kay

(12) found decreased concentrations of organic acid-soluble P in the blood cells of rats with rickets induced by a high Ca, low P diet; this decrease was prevented, and the lowered values restored to normal, by the administration of cod liver oil or viosterol. In the whole blood of infants with healing rickets, Freudenberg (13) found slightly higher values for organic P than he found in the bloods of the same infants before the rickets was treated. Stearns and Warweg (14) studied the distribution of P in the bloods of three children with late rickets, and noted significant increases of ester P in the blood cells during healing. In 1937 Bakwin *et al.* (15) reported that in rachitic infants they found a reduction in the organic acid-soluble P of the blood cells which was partly accounted for by decreases in the readily acid-hydrolyzable P (adenosinetriphosphate) and partly by decreases in the fraction not hydrolyzable by bone enzyme; the latter fraction was thought to represent diphosphoglycerate.

The studies here reported deal with the organic acid-soluble P, diphosphoglyceric acid, and adenosinetriphosphate in the cells, and the inorganic P in serum and cells, of the bloods of normal and rachitic rats and of rachitic rats following (1) the administration of irradiated ergosterol, (2) the addition of phosphate to the rachitogenic diet, and (3) fasting.

Methods

Collection of Blood—With the rat tied on its back, under light ether anesthesia, both internal jugular veins were exposed and cut. The flowing blood was drawn into a medicine dropper and transferred to a small vial containing glass beads. Bloods of from two to five animals (depending on their size and the amount of blood obtained from each) were thus collected, pooled in the same vial, defibrinated by shaking with the beads, and then filtered through gauze. In a few experiments crystalline heparin was used as an anticoagulant.

The volume of packed cells in whole blood was determined by the method of Guest and Siler (16).

Inorganic and total acid-soluble P was determined by the method of Fiske and Subbarow (17) with modifications previously described (18). In some instances in which the concentration of inorganic P was very low, the more sensitive method of Kuttner

and Lichtenstein (19) modified by Bodansky (20) was used. Diphosphoglyceric acid was determined as glyceric acid by the method of Rapoport (21). Adenosinetriphosphate was determined according to Lohmann (22). The figure for easily hydrolyzable P, obtained after the trichloroacetic acid filtrate was heated with $N H_2SO_4$, was multiplied by $3/2$ to give the value designated adenosinetriphosphate P. The concentrations of organic acid-soluble P and of its fractions in the blood cells were calculated in each instance from determinations made on whole blood, without correction for the slight traces of organic acid-soluble P which might have been present in the blood serum. The concentration of inorganic P in the cells was calculated from values determined on the whole blood and serum, by means of the cell volume per cent.

Line tests were performed as described by McCollum *et al.* (4), and readings were recorded from 0 to ++++ according to the notation of Bills and McDonald (23).

Results

Normal Controls—In Table I are listed data on the distribution of acid-soluble P in fourteen samples of blood obtained as described above from rats fed a normal diet. In addition to the blood samples listed, others have been examined, with less complete analyses of the several fractions. The further data thus obtained agree with those cited, but are omitted from Table I for the sake of brevity. The values for inorganic P in the serum and cells agree with those commonly reported in current literature. The values for organic acid-soluble P in the cells agree with those reported by Kay (12). In the bloods of rats weighing over 100 gm. the diphosphoglycerate P makes up about half of the organic acid-soluble P, as has been reported for normal rabbit and human blood (18, 24), and the adenosinetriphosphate P amounts to approximately one-fifth of the organic acid-soluble P, in agreement with values reported by Kerr and Daoud (25). In the bloods of the younger rats the values for organic acid-soluble P and diphosphoglycerate in the cells tended to be higher.

Rickets—Rats initially weighing 50 to 60 gm. were fed the Steenbock and Black (26) diet, Ration 2965, during periods from 3 to 50 days. Some were bled after 3, 7, and 14 days on this diet,

in order to determine the changes occurring in the blood during the early stages of the development of rickets. After 21 days on this diet, those that weighed less than 65 gm., or that had gained less than 10 gm. during this period, were discarded. The others, showing growth above this minimum requirement, were either bled at the various times designated in Table II, or were used for

TABLE I
Distribution of Acid-Soluble P in Samples of Blood from Normal Rats

Weight	No. of rats bled for each sample	Volume of packed cells	Whole blood				Serum inorganic P	Cells			
			Inorganic P	Organic acid-soluble P	Diphosphoglycerate P	Adenosinetriphosphate P		Inorganic P	Organic acid-soluble P	Diphosphoglycerate P	Adenosinetriphosphate P
gm.		per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
56-64	5	35.0	5.9	27.7	17.0	3.8	7.3	3.3	79.2	48.5	10.9
	5	34.3	5.7	26.5	15.3	3.8	6.9	3.3	77.1	44.5	10.9
	4	35.7	6.2	25.9	13.9	3.6	7.5	3.6	72.4	38.8	10.0
82-96	3	37.0	7.3	24.2	12.9	6.2	8.5	5.1	65.4	34.7	16.7
	3	36.5	7.3	27.0	14.0	5.7	8.2	5.8	73.8	38.3	15.7
	3	37.3	7.5	26.7	13.9	5.7	8.8	5.2	71.5	37.2	15.2
102-125	3	40.2	7.6	26.7	15.3	5.3	9.0	5.5	66.3	38.1	13.2
	3	39.8	7.1	26.1	15.3	5.2	8.2	5.5	65.7	38.5	13.0
	3	38.5	6.8	26.8	14.9	5.3	8.0	4.8	69.6	38.7	13.8
120-140	2	37.7	5.9	24.6	12.3	6.0	7.1	3.8	65.2	32.7	15.9
	2	35.3	5.6	23.6	12.1	5.2	6.5	4.1	66.8	34.3	14.7
	2	37.7	5.0	25.0	12.5	5.2	6.0	3.3	66.4	33.1	13.7
	3	34.5	5.6	23.3	12.2	4.9			67.4	35.3	14.1
	2	42.9	5.8	28.9	14.9	5.8	6.9	4.2	67.3	34.8	13.6

the healing experiments listed in Tables III and IV. In the discussion that follows, the latter are designated 21 day, or 50 day, rachitic rats, according to the length of time they received the rachitogenic diet without other treatment.

In the blood samples obtained at varying times after the rats were given the rachitogenic diet, decreases were found in all the fractions of acid-soluble P listed in Table II. The decrease of

inorganic P in the serum at 3 and 7 days was about the same as that found by Warkany (27) during similar periods. The decrease of inorganic P and adenosinetriphosphate in the cells ap-

TABLE II

Distribution of Acid-Soluble P in Samples of Blood from Rats Fed Steenbock and Black Rachitogenic Ration 2965 during Periods from 3 to 50 Days

Rachitogenic diet period	No. of rats bled for each sample	Whole blood						Serum inorganic P	Cells			
		Volume of packed cells	Inorganic P	Organic acid- soluble P	Diphosphogly- cerate P	Adenosinetri- phosphate P	Inorganic P		Organic acid- soluble P	Diphosphogly- cerate P	Adenosinetri- phosphate P	
days		per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
3	3	36.5	3.5	25.5	15.3	3.2	4.6	1.7	70.0	42.0	8.6	
3	4	36.6	3.5	26.0	14.6	2.8	4.8	1.4	71.1	39.8	7.6	
3	4	37.7	3.6	21.9	11.3	3.2	4.8	1.7	58.1	30.1	8.6	
7	4	39.0	2.0	25.0	13.7	2.9	2.3	1.6	64.0	35.1	7.5	
7	4	40.3	2.4	25.7	13.6	2.8	3.0	1.4	63.8	33.6	7.0	
14	4	40.1	1.3	17.4	9.2	2.0	1.5	1.0	43.3	23.1	5.1	
14	4	42.0	1.5	19.4	10.7	2.1	1.9	1.0	46.3	25.4	5.0	
21	4	35.1	1.5	15.5	5.9	2.9	1.7	1.1	44.0	16.7	8.3	
21	4	40.4	2.1	18.2	8.5	3.2	2.5	1.4	45.0	21.0	7.8	
23	5	42.2	1.8	18.8	10.1				44.4	24.0		
23	3	42.5	1.8	22.6	11.2				53.3	26.4		
23	3	35.0	2.6	18.6	9.3				53.3	26.5		
23	3	39.9	2.0	18.6	12.2				46.5	30.5		
23	3	41.9	2.2	21.1	10.9				50.3	26.0		
23	3	42.9	1.8	19.5	10.3	3.6			45.5	23.9	8.4	
24	3	41.6	1.8	16.4	8.6				39.5	20.7		
24	4	38.8	1.3	18.0	8.2	3.8	1.5	0.9	46.3	21.1	9.8	
27	5	38.4	1.5	18.9	9.0	2.2			49.3	23.3	5.8	
28	4	37.6	1.6	20.0	10.8	2.8			53.1	28.6	7.5	
28	4	38.3	1.6	20.0	9.5	2.8			52.3	24.7	7.3	
31	3	42.4	1.8	16.9	8.4				35.1	19.9		
35	3	43.0	1.7	16.0	6.7	3.0	2.0	1.5	37.3	15.6	6.9	
46	3	46.1	1.3	16.0	10.2	2.4	1.4	1.1	34.8	22.2	5.3	
50	3	44.2	1.6	15.7			1.8	1.3	35.6			

peared to be more abrupt. At 7 days the diphosphoglycerate had decreased only slightly, but at 14 days this fraction also was markedly reduced. After 14 days the inorganic P in both serum

TABLE III

Distribution of Acid-Soluble P in Samples of Blood from 21 Day Rachitic Rats, after Healing Was Induced by Administration of Single Doses of Irradiated Ergosterol Equivalent to 5 Rat Units

Days after irradiated ergosterol	No. of rats bled for each sample	Whole blood					Serum inorganic P	Cells				Line tests
		Volume of packed cells	Inorganic P	Organic acid-soluble P	Diphosphoglycerate P	Adenosinetriphosphate P		Inorganic P	Organic acid-soluble P	Diphosphoglycerate P	Adenosinetriphosphate P	
		per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
3	4	38.8	2.5	20.2	15.8	2.3	4.4	-0.4	52.1	40.6	5.8	0 0 ++ ++
5	3	41.4	2.1	25.2	17.7	3.2	3.2	0.2	60.8	42.8	7.8	+± ++ ++ ++
5	3	37.5	2.4	24.0	16.7	3.2	3.9	-0.1	64.1	44.5	8.6	0 ++ ++
7	4	36.4	2.4	22.1	15.7	2.4	3.8	-0.1	60.8	43.3	6.6	++ ++ ++± ++±
7	4	36.9	1.9	21.8	16.0	1.9	3.2	-0.2	58.9	43.3	5.1	0 ++ ++ ++
10	4	40.7	2.4		15.1	4.9	3.4	1.0		37.1	12.0	+± ++ ++± ++±
10	3	44.4	2.6	25.5	15.0	5.2	4.2	0.6	57.4	33.8	11.8	++± ++± ++± ++±
10	3	30.5	2.0	17.7	12.8	4.4	2.5	0.7	58.1	41.9	14.3	+± +± +++
10	3	41.5	2.1	22.5	15.1	5.9	3.1	0.8	54.2	36.4	14.1	++ ++± ++±

TABLE III—*Concluded*

Days after irradiated ergosterol	No. of rats bled for each sample	Whole blood					Serum inorganic P	Cells				Line tests
		Volume of packed cells	Inorganic P	Organic acid-soluble P	Diphosphoglycerate P	Adenosinetriphosphate P		Inorganic P	Organic acid-soluble P	Diphosphoglycerate P	Adenosinetriphosphate P	
		per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
22*	4	42.5	2.3	23.0	12.5	5.2	3.3	1.0	54.2	29.4	12.2	++ ++++± ++++± Lost
22*	5	42.8	2.5	24.0	12.9	5.1	3.4	1.4	56.1	30.2	12.0	++ ++ ++ ++± +++++

* These rats were given a second dose of 5 rat units of irradiated ergosterol on the 10th day.

and cells was reduced to low concentrations, but the ratio of cell to serum values was about the same as that found in normal rats. All values remained relatively constant after 14 days. The concentration of organic acid-soluble P in the cells decreased to about two-thirds of the average normal concentration, in agreement with Kay's findings (12). This decrease was accounted for in the adenosinetriphosphate and diphosphoglycerate fractions, confirming the surmise of Bakwin *et al.* (15).

Healing Rickets, Induced by Irradiated Ergosterol—In Table III are listed data on blood samples from 21 day rachitic rats that were bled at varying times after they were given single doses of irradiated ergosterol, calculated to be equivalent to 5 rat units, U.S.P. The preparation used was drisdol, a solution of irradiated ergosterol in propylene glycol; this was diluted 1:100 in water and 0.14 cc. of the diluted solution was given by mouth. Two groups of rats were given a second dose on the 10th day, and then were bled on the 22nd day. The symbols in the last column of Table III show the results of the line tests in each of the rats whose bloods were pooled for each set of analyses. The dose of irradiated

ergosterol thus given induced at least ++ healing in the epiphyses after 10 days. The inorganic P in the serum increased somewhat above the rachitic level, but remained low at around 3.5 mg. per 100 cc., as previously observed by Warkany (27). The inorganic P in the cells, on the other hand, decreased to very low concentrations in the 5 and 7 day samples and increased only slightly in the 10 and 22 day samples; even in the latter the ratio of cell to serum values remained far below normal. Since the inorganic P concentrations increased in the serum and decreased in the cells, the values for the whole blood appear to be very little higher than those found in the untreated rachitic rats. The concentration of organic acid-soluble P in the cells was approximately normal in the 5 and 7 day samples, but tended to be slightly lower in the 10 and 22 day samples. The concentration of diphosphoglycerate P in the cells of the 3, 5, and 7 day samples was nearly double that found in the bloods of the untreated rachitic rats, and even considerably higher than in the normal rats. In the 10 day samples this value had decreased to about normal, and in the 22 day samples it was slightly below the average normal level. The concentration of adenosinetriphosphate P in the cells of the 3, 5, and 7 day samples was very little changed from the rachitic values, but rose to approximately normal in the 10 and 22 day samples.

Healing Rickets, Induced by Addition of Phosphate to Rachitogenic Diet and by Fasting (Table IV)— NaH_2PO_4 was added to the diets of 21 day rachitic rats to make the Ca:P ratio 1:0.5, and groups of these rats were bled at 5, 10, and 15 days after the change in the diet. In the 5 day samples the inorganic P was found increased in the serum and slightly decreased in the cells, compared with the values for the rachitic rats. In the 10 and 15 day samples, however, the inorganic P in the cells had increased somewhat. The concentration of organic acid-soluble P in the cells was somewhat low (around 50 mg. per 100 cc.) in all samples. High normal values for the concentration of diphosphoglycerate P in the cells were found in the 5 day samples, but lower values were found in the 10 and 15 day samples.

Groups of 50 day rachitic rats, given this diet with P added to make the Ca:P ratio 1:0.5, were bled after 5, 9, and 15 days. The changes in the distribution of acid-soluble P in these bloods were

TABLE IV
Distribution of Acid-Soluble P in Blood of 21 Day and 50 Day Rachitic Rats after Addition of Phosphate to Diet, and in Blood of 21 Day Rachitic Rats after Brief Fast

Treatment of groups	Days after change in diet	No. of rats bled for each sample	Whole blood					Serum inorganic P	Cells					
			Vol. of urine of packed cells	Inorganic P	Organic acid-soluble P	Di-phosphoglycerate P	Adenosinetriphosphate P		Inorganic P	Organic acid-soluble P	Di-phosphoglycerate P	Adenosinetriphosphate P		
													mg. per 100 cc.	mg. per 100 cc.
21 day rachitic; then P added to make Ca: P 1:0.5	5	4	36.7	2.5	18.3	13.2		3.6	0.6	50.0	35.9			
	5	4	40.5	3.0	20.3	15.2		4.6	0.6	50.1	37.6			
	10	4	35.4	2.7	19.3	12.3		3.7	0.9	54.4	34.7			
	10	4	39.3	3.3	21.0	13.1		4.7	1.2	53.3	33.3			
	15	4	41.7	2.4	20.5	13.3		3.4	1.1	49.1	31.8			
50 day rachitic; P added to make Ca: P 1:0.5	15	4	43.0	2.1	22.8	13.4		2.9	1.0	52.9	31.2			
	5	3	44.5	2.3	25.1	13.8				56.4	31.0			
	5	3	38.4	2.5	20.0	11.7				52.1	30.5			
	9	3	41.1	2.9	20.7	9.9		4.5	0.5	50.3	24.2			
	9	3	33.8	2.7	18.2	7.4		3.9	0.5	53.9	21.8			
21 day rachitic; P added to make Ca: P 1:1.1	15	3	41.6	3.4	21.5		3.4	5.7	0.1	51.7				8.2
	2	4	42.2	4.7	24.2		2.5	7.4	1.0	57.3				5.9
	2	4	43.0	3.8	23.7	13.0	2.2			55.0	30.3			5.1
	2	4	40.8	4.3	21.3	11.6	2.0			52.2	28.5			4.9
	10	4	41.0	7.6	27.1		5.0	9.7	4.6	66.1				12.2
21 day rachitic; then fasted	14	4	39.2	5.7	25.8	14.3	4.2	7.0	3.7	65.8	36.6			10.7
	1½	4	41.9	7.2	22.9	14.7	2.5	11.6	1.1	54.7	35.1			6.0
	3	4	46.1	5.8	20.9		2.8	9.7	1.1	45.2				6.1

essentially the same as those found in the 21 day rachitic rats described above.

In some groups of 21 day rachitic rats NaH_2PO_4 was added to the rachitogenic diet to make the Ca:P ratio 1:1.1; the rats were bled after 2, 10, and 14 days. The inorganic P in the serum, determined in two of these samples, at 2 and 10 days, respectively, after the change in the diet, was found increased to a normal concentration and higher, in agreement with the findings of Karelitz and Shohl (28) under similar conditions. In the 2 day samples the organic acid-soluble P and diphosphoglycerate P in the cells attained low normal concentrations, but the adenosinetriphosphate remained low. In the 10 and 14 day samples all values were essentially normal.

Two groups of 21 day rachitic rats were fasted for $1\frac{1}{2}$ and 3 days, respectively, and then were bled. While the inorganic P in the serum of these bloods was high, as previously reported by Wilder (6), the inorganic P in the cells remained low. Likewise the concentration of adenosinetriphosphate in the cells remained low, while the organic acid-soluble P increased to around 50 mg. per 100 cc. and the diphosphoglycerate attained a normal level after $1\frac{1}{2}$ days.

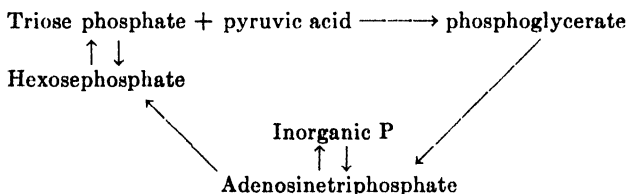
DISCUSSION

These experiments offer further evidence that diphosphoglyceric acid in the blood cells acts as an important transport substance in the intermediate phosphorus metabolism of the body, a rôle postulated for this compound in previous communications (18, 21). The relationship of these chemical changes in the blood to the development and healing of rachitic lesions in the bones is not clear. While Robison and his coworkers (29) have suggested that certain phosphoric esters may be concerned in ossification, it is not definitely known whether phosphorus must pass through the ester stage before it can be assimilated by bone. It may be said, however, that under the conditions of the experiments cited here, a marked decrease in concentration of diphosphoglycerate in the blood cells can be correlated with the development of rickets, and that healing is preceded by increases of phosphoglycerate in the cells. The changes of inorganic P and adenosinetriphosphate in the blood, previously demonstrated by other investigators in

similar experiments, are closely interrelated with the changes of phosphoglycerate.

The finding that the changes in phosphorus distribution in the blood were similar when healing of rickets was induced in different ways suggests that the same chemical reactions were called into play by the several procedures. Those procedures (administration of irradiated ergosterol, addition of P to the diet, and fasting) all make for greater availability of phosphorus for purposes of transfer in the body, and the mechanism of transfer involves a series of chemical changes which are closely linked with various metabolic processes in addition to those concerned with ossification.

The modern theory of glycolysis, developed by Embden, Meyerhof, Parnas, and others (reviewed by Robison (30)) offers some help to the understanding of these changes. According to current concepts, P is transferred from adenosinetriphosphate to glucose, to yield hexosediphosphate which is then split into two triose phosphates. By reaction with pyruvic acid, these produce phosphoglyceric acid, which in turn is broken down and its P transferred to adenylic acid, thus reproducing the adenosinetriphosphate. The reaction between pyruvic acid and triose phosphate which yields phosphoglyceric acid is linked with an esterification of inorganic P and adenylic acid; this is able to compensate losses due to phosphatase action (Dische (31)). These reactions may be represented by the following simplified diagram.



With this scheme of reactions in mind, an attempt can be made to visualize the reactions taking place in the blood during the healing of rickets. The increase in available phosphorus effected by the administration of irradiated ergosterol, fasting, or the addition of P to the diet is followed by increased synthesis, or esterification, in the cells, the P first passing over to adenosinetriphosphate and

then to phosphoglycerate, in a series of reactions similar to those which have been described for glycolysis. The concentration of phosphoglycerate in the blood cells increases first to high values, while the inorganic P and adenosinetriphosphate concentrations remain low. The low concentrations of inorganic P and adenosinetriphosphate can be explained by an increased rate of the reactions going in the direction of phosphoglycerate synthesis. After a time, when equilibrium is reached, the phosphoglycerate decreases and the inorganic P and adenosinetriphosphate increase. This equilibrium may be reached when a balance is attained between the available supply of P and the demand for its transfer to different parts of the body.

SUMMARY

In rats given a high Ca, low P rachitogenic diet, the development of rickets was associated with decreases first of inorganic P and of adenosinetriphosphate and then of diphosphoglyceric acid in the blood cells. Healing of rickets, induced in these animals in different ways, was preceded by increases first of diphosphoglyceric acid and then of adenosinetriphosphate and inorganic P in the blood cells. The interrelationship of the changes of inorganic P, adenosinetriphosphate, and diphosphoglycerate is discussed, and it is suggested that the conversions of these substances in the *blood cells* constitute important steps in the mechanism of transport of phosphorus in the body.

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THE ENDOCRINE CONTROL OF LIPID METABOLISM IN THE BIRD*

II. THE EFFECTS OF ESTRIN ON THE BLOOD LIPIDS OF THE IMMATURE DOMESTIC FOWL

By F. W. LORENZ, I. L. CHAIKOFF, AND C. ENTENMAN

*(From the Division of Poultry Husbandry of the College of Agriculture, Davis,
and the Division of Physiology of the Medical School,
University of California, Berkeley)*

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The striking changes that appear in the lipid content of blood and liver in association with the onset of maturity have been the subject of previous reports from this laboratory (1, 2). In order to determine the mechanism whereby these changes are brought about, studies have been undertaken on the effects of the administration of hormones on the lipid metabolism of the bird. It was shown that prolonged administration (21 days) of pregnant mare serum, which contains the gonad-stimulating principle, led to a rise in all lipid constituents of the blood, although neutral fat was principally affected (3). So far as the blood lipids were concerned, the effects produced by the administration of the pregnant mare serum were similar to those observed in birds in which laying had spontaneously occurred, but the liver lipids, the content of which rises in the laying state, failed to respond to the hormone administration. The question now arises whether its effect here is secondary; *i.e.*, brought about through its stimulating effect upon ovarian activity. To determine this, it became necessary to investigate the influence of the ovarian hormones themselves upon lipid metabolism. It is shown in the present investigation that estrin in suitable doses can double the lipid content of the blood in as short a time as 24 or 48 hours after its administration. It would thus appear that the increased activity of the ovary is

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the mechanism whereby pregnant mare serum raises the blood lipids in birds.

EXPERIMENTAL

An estrogenic concentrate was prepared from pregnant mare urine by the method of Beall and Marrian (4). The toluene extract of concentrated, acid-hydrolyzed urine was extracted with peroxide-free ether, washed five times with half saturated aqueous sodium carbonate solution and four times with water, and evaporated to dryness in an atmosphere of CO₂. This concentrate, which was assayed¹ on ovariectomized rats, contained 25,000 rat units of estrin per cc. For injection the material was diluted 1:25 with corn oil.

Single comb white Leghorn chicks of the Poultry Division stock were used in this investigation. The female birds were immature and 74 days old, the male birds 66 days old, when injections were started. They were fed the stock diet, the composition of which has been recorded elsewhere (2). The estrogenic concentrates were administered intramuscularly in amounts varying between 500 and 3000 rat units. For control studies, birds were injected with the same quantity of corn oil as was used for diluting the hormone concentrate.

Blood lipid examinations were made at various intervals, in most cases at 24 or 48 hours after the hormone treatments had begun. In the 24 hour cases the total dose was divided into three portions and all injections made within 1 day; in those of 48 hours the total dose was divided into six equal portions and all these were injected during 2 successive days. Injections were made into the pectoral muscle at 8 a.m., 12 m., and 4 p.m. Blood samples were obtained by heart puncture between 8 a.m. and 10 a.m. on the morning following the last injection, and before this the birds had been deprived of food for 16 hours.

Results

The lipid levels of the blood of the control group are shown in Table I, while the results obtained in birds treated with the hormone are recorded in Tables II to IV. Table II contains the

¹ We are indebted to Dr. H. H. Cole of the College of Agriculture at Davis for assays of the hormone used in this study.

values found in immature *female* birds examined at 24 and 48 hours after initiation of estrin treatments. Table III shows the effects of estrin on *male* birds.

Immature Female Birds—24 hours after the estrin treatments were begun, striking changes in the levels of the blood lipids had already occurred (Table II). The effects obtained with the larger

TABLE I
Whole Blood Lipids of Control Birds

Each bird received 1 to 3 cc. of corn oil intramuscularly.

Bird No.	Sex	Weight gm.	Age days	Oil injected cc.	No. of injections	Interval* hrs.	Cholesterol			Total fatty acids mg. per 100 cc.	Phospholipid mg. per 100 cc.	Total lipid mg. per 100 cc.	Residual fatty acid† mg. per 100 cc.
							Total	Free	Ester				
							mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.				
53	F.	580	74	3	3	24	121	90	31	317	281	438	106
54	"	630	74	3	6	48	119	92	27	330	177	449	191
55	"	650	74	3	3	24	112	70	42	287	251	399	88
56	"	840	74	3	6	48	116	76	40	350	292	466	125
75	"	550	73	1	1	12	108	89	19	285	281	393	83
63	"	640	73	1	1	3	78	75	3	358	331	436	134
64	"	610	73	1	1	3	128	103	25	334	302	462	114
84	M.	700	66	3	6	48	135	84	51	311	295	446	76
85	"	810	66	3	6	48	128	95	33	280	257	408	84
86	"	750	66	3	6	48	111	89	22	245	232	356	73
87	"	630	66	3	6	48	113	106	7	320	238	433	155
88	"	750	66	3	6	48	122	110	12	356	259	478	174
89	"	670	66	3	6	48	141	106	35	361	292	502	140

* Time between the first injection and the time blood was taken for lipid analysis.

† Fatty acids other than those in combination with phospholipids and cholesterol.

doses, *i.e.* 2000 and 3000 units per bird, were greater than those observed with 1000 or 500 units, although increases varying from 70 to 100 per cent in the concentration of total lipid were found with the two latter dosages. All lipid constituents were not equally affected. While definite rises were noted in phospholipids and to a lesser degree in total cholesterol, the lipid constituent that showed the most marked response was neutral fat.

At the 48 hour interval (Table II) the values observed for total lipids were higher than those found at the earlier interval when doses of 1000, 2000, and 3000 units per bird were employed. Here again the most pronounced change occurred in neutral fat, al-

TABLE II

Effect of Varying Doses of Estrin upon Whole Blood Lipids of Female Birds

The portions of estrin were injected at 8 a.m., 12 m., and 4 p.m. The preparation contained 1000 rat units per cc. of corn oil. Each bird was 74 days old at the time injections were started.

Bird No.	Total dose	Weight	Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
			Total	Free	Ester				

Determinations made 24 hrs. after first injection. Total doses were divided into three equal portions

	rat units	gm.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
37	3000	670	157	101	56	780	443	937	442
38	3000	620	159	115	44	919	489	1078	566
41	2000	740	143	108	35	640	390	783	352
42	2000	630	185	134	51	1015	529	1200	624
45	1000	730	150	111	39	678	450	828	348
46	1000	510	164	106	58	577	364	741	291
49	500	660	176	98	78	471	334	647	190
50	500	520	151	109	42	608	416	759	298

Determinations made 48 hrs. after first injection. Total doses were divided into six equal portions

39	3000	700	232	174	58	1729	724	1961	1202
40	3000	560	198	133	65	1086	551	1284	670
43	2000	730	177	118	59	774	413	951	454
44	2000	600	227	155	72	1148	525	1375	743
47	1000	620	219	142	77	1041	480	1260	663
48	1000	690	200	135	65	952	443	1152	608
51	500	610	196	155	41	379	240	575	188

though free and esterified cholesterol and phospholipids increased considerably.

Male Birds—The lipid content of the blood of male birds (controls) that received 3 cc. of corn oil in equally divided doses over a period of 48 hours was 356 to 502 mg. per cent (Table I). In three birds that had received 2000 rat units of estrin contained

TABLE III

Effect of Estrin on Whole Blood Lipids of Male Birds

Each bird received 2000 rat units in 2 cc. of corn oil intramuscularly. All birds were 66 days old at the time injections were started.

Bird No.	Weight	No. of injections	Interval	Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
				Total	Free	Ester				
	gm.		hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
78	650	3	24	121	100	21	529	432	650	224
80	600	3	24	159	130	29	600	522	759	229
82	950	3	24	145	99	46	553	375	698	268
79	700	6	48	183	120	63	890	511	1073	502
81	720	6	48	188	137	51	899	400	1087	604
83	800	6	48	169	126	43	857	408	1026	552

TABLE IV

Time of Onset of Blood Lipid Changes Following Injection of 3000 Rat Units of Estrin into Immature Female Birds

Whole blood was used for the analyses. The dose recorded was given as a single intramuscular injection in 1 cc. of corn oil. All birds were 73 days old at the time the injections were started.

Bird No.	Weight	Interval after injection	Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
			Total	Free	Ester				
	gm.	hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
69	630	3.2	112	86	26	340	267	452	143
70	660	3.2	110	86	24	326	274	436	124
61	720	3.4	96	80	16	366	191	462	226
62	610	3.5	137	84	53	302	266	439	85
77	600	6.3	108	76	32	296	234	404	115
66	650	6.3	95	83	12	410	262	505	226
67	610	6.4	102	86	16	347	270	449	154
68	610	6.5	104	89	15	300	292	404	93
71	680	12	121	98	23	590	390	711	312
72	690	12	109	96	13	569	456	678	254
73	535	12.5	97	82	15	477	283	574	276
74	630	12.5	139	88	51	607	400	746	301

in 3 cc. of corn oil that had been administered in six equally divided doses over a period of 48 hours, the blood contained 1026, 1073, and 1087 mg. per cent of total lipids (Table III). When the same doses were given in three equal parts over a period of 24 hours, the blood examined at the end of this period contained between 650 and 759 mg. per cent of total lipids. As noted above, all lipid constituents shared in this rise, but the most pronounced effect was found in neutral fat.

Time of Onset of Lipid Changes in Blood—In view of the positive effects of estrin, it became of interest to determine the time of onset of these lipid changes. The maximum dose previously used, namely 3000 rat units per bird, was administered to twelve immature female birds in single injections and the blood examined at 3, 6, and 12 hours thereafter (Table IV). The results show that no effects were obtained with this large dose at the 3rd and 6th hour after the hormone administration, but by the 12th hour significant rises had occurred. The two lipid constituents that responded during this short interval were neutral fat and phospholipids; none of the cholesterol fractions had yet shown a change at this time.

DISCUSSION

Lipid metabolism in the bird is unique in that it is under the control of the ovarian hormones. This has now been shown by three sets of observations: (a) the enormously high lipid levels in the blood of birds in the active laying state (1), (b) the doubling of the lipid content of the blood produced by experimental stimulation of the ovaries of immature birds by pregnant mare serum (3), and (c) the marked rise in blood lipids that follows the introduction of an exogenous source of the ovarian hormone, estrin.

The rapidity of the response of the blood lipids to estrin—within 12 hours after the hormone injection—is indeed worthy of note, for in this respect estrin differs from pregnant mare serum, which required prolonged administration before the blood lipids rose (3).

Even more striking than its effect on the female bird is the rise in blood lipids of the male that follows the intramuscular injection of estrin. It was previously shown here that when the gonad-stimulating principle of pregnant mare serum was injected into

immature female birds, a rise in blood lipids occurred if an oviduct size of at least 10 gm. had been attained (3). The rise in blood lipids now produced by estrin in the male bird shows that the relation between the level of the blood lipids and the oviduct size is not causal. The size of the oviduct is probably an index of ovarian activity under the influence of the pregnant mare serum and when enough of the ovarian hormone has been secreted to stimulate the oviduct to a growth of at least 10 gm., this amount of hormone is also enough to increase the blood lipids. It would therefore seem that estrin has a *direct* effect on lipid metabolism of the bird and that the rise in blood lipids does not require the intervention of egg production or of the accessory sex organs such as the oviduct.

SUMMARY

1. Estrin raises the blood lipids of the immature female bird. After the intramuscular injection of 3000 rat units, the total lipid content of the blood is more than doubled.

2. This effect on the immature female bird is obtained rapidly; *i.e.*, within 12 hours after a single injection of 3000 rat units of estrin.

3. The blood lipids of the male bird also respond to estrin. Total lipid values well over 1000 mg. per 100 cc. of whole blood were found after the injection of 2000 rat units of the hormone.

4. All lipid constituents, namely cholesterol, phospholipids, and neutral fat, share in the rise, but the most pronounced effect is produced upon neutral fat.

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A METHOD FOR THE DETERMINATION OF SMALL QUANTITIES OF ASCORBIC ACID AND DEHYDROASCORBIC ACID IN TURBID AND COLORED SOLUTIONS IN THE PRESENCE OF OTHER REDUCING SUBSTANCES

By OTTO A. BESSEY*

(From the Harvard Dental School and the Departments of Pathology and Biological Chemistry, Harvard Medical School, Boston)

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Most of the chemical methods which have been proposed for the estimation of ascorbic acid have been based upon the principle that, under suitable experimental conditions, ascorbic acid more or less specifically and quantitatively reduces certain colored reagents (1). The simplicity of manipulation plus the relatively close correlation between the vitamin C content of plant and animal tissues (biologically assayed) and the titration value against 2,6-dichlorophenol indophenol has led to extensive use of this reagent (2-4).

Although the titration procedure has been very useful and quite satisfactory for most plant tissue and some animal tissues, there are several limitations and hazards involved in its general use. The process of titration, with usual end-point detection, is not precise for the determination of small quantities of ascorbic acid. It has been impossible to analyze extracts satisfactorily which are highly colored, such as those from beets, berries, and certain leaves. Turbid solutions lead to high end-points. The presence in most animal tissues and fluids of substances other than ascorbic acid, which slowly reduce the dye, results in high and indistinct end-points. Another important objection has been the difficulty of defining the conditions under which the extracts and reagents are most stable and the reactions quantitative. The need of frequent standardization of the dye solution is inconvenient.

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By direct titration only the reduced form of the vitamin is estimated. The question has arisen as to how much of the reversibly oxidized (dehydroascorbic acid) but biologically active form may be naturally present in some tissues, or present in the extracts as a consequence of the method of extraction. The increased titration value after treatment with hydrogen sulfide has been generally used as a measure of dehydroascorbic acid, although the practical optimum conditions for this reaction have not been clearly determined (5-8).

Many new problems of ascorbic acid chemistry and metabolism would become subject to study if a more precise analytical method were available.

Mindlin and Butler (9) have recently suggested a photoelectric method for ascorbic acid determinations in plasma. However, this procedure is limited to use with clear, colorless filtrates and solutions. This paper describes a development and modification of the photoelectric method suitable for general use and which overcomes most of the objections and limitations of the titration technique. A precision of 1 to 3 per cent on samples containing 6 to 20 micrograms of ascorbic acid or dehydroascorbic acid is possible by this new procedure.

A 3 per cent metaphosphoric acid extract of the unknown is buffered to pH 3.5 to 3.7. The decrease in the concentration of the colored, oxidized form of the dye in an aqueous solution of 2,6-dichlorophenol indophenol by the addition of a quantity of the buffered extract insufficient to reduce the dye completely is measured by means of a photoelectric colorimeter (10).¹ Dehydroascorbic acid is represented by the difference between the above determination and one carried out on an aliquot of the same buffered extract after treatment with H_2S and subsequent elimination of the highly reducing sulfide by a stream of wet nitrogen. The instrument is operated in a manner to correct automatically for turbidity or extraneous pigments. As pointed out by Mindlin and Butler, observations of the rate of fading of the color with the time permit the detection and correction for the effects of interference from most substances, naturally occurring. "The method

¹ The design and discussion of the principle of operation of this type of instrument have been published by Evelyn (10).

eliminates the subjective reading of an end-point and the need of an accurately standardized dye solution" (9).

Determination

Apparatus and Reagents—The Evelyn photoelectric colorimeter with Filter 520, as previously described, has been used (9, 10). However, any equivalent instrument should suffice.

The 2,6-dichlorophenol indophenol solution is made by dissolving approximately 8 mg. of the crystals in 500 cc. of warm distilled water, cooling, and filtering. Keep in the ice chest when not in use. The dye solution slowly changes, but remains satisfactory for use for about 10 days.

TABLE I

*Rate of Conversion of Metaphosphoric Acid to Orthophosphoric Acid;
3 Per Cent Solution*

Temperature	Orthophosphoric acid, mole per cent			
	0 hr.	24 hrs.	3 days	14 days
°C.				
5	8.1	8.2	8.2	9.6
25	8.1	11.6	17.4	
38	8.1	16.1	26.3	

Metaphosphoric acid solution (6 per cent) is made by dissolving the clear sticks of glacial metaphosphoric acid in distilled water and filtering. This stock is left in the ice box and 3 per cent acid made as required. Metaphosphoric acid slowly reacts with water to form the more acid orthophosphate. The rate of this change at different temperatures has been measured (by analysis for orthophosphate by the method of Fiske and Subbarow (11), Table I). The acid remains satisfactory for use after 15 days at 5°.

Citrate buffer. 21.0 gm. of citric acid are dissolved in 200 cc. of 1.0 N (carbonate-free) sodium hydroxide and diluted to a volume of 250 cc. The pH of various quantities of this buffer with 3 per cent metaphosphoric acid is shown in Table II. This table is useful for the preparation of the proper blank solution as required in the procedure.

Procedure—A representative portion of the tissue (0.5 to 2 gm.) is

weighed and an extract prepared, as previously described by Bessey and King (2) except that 3 per cent metaphosphoric acid is used as the extracting reagent. The size of the sample and the final volume of the extract should be selected so that each 50 cc. of extract contains 100 to 450 micrograms of ascorbic acid. In most cases, by this method the volume of the solids of the sample is insignificant compared with the total volume of the extract and it becomes more practical and nearly as precise to use an accurately measured volume of extracting medium and dispense with the procedure of washing.

To 25 cc. of the centrifuged extract are added 7 cc. of the citrate buffer, which brings the pH to 3.5 to 3.6 for most plant tissues.

TABLE II

pH of Metaphosphoric Acid-Citrate Buffer Mixtures Measured with Glass Electrode at 25°

25 cc. of 3 per cent metaphosphoric acid.

Citrate buffer	pH of mixture
cc.	
12	4.02
10	3.90
9.0	3.77
8.0	3.67
7.0	3.48
6.0	3.30
5.0	3.02

Only 6 cc. of the buffer should be used for most animal tissues.² The concentration of the colored oxidized form of the dye remaining after reaction with the ascorbic acid of the sample is determined as follows: With the photoelectric colorimeter set so that the galvanometer reads 100 for a tube of distilled water, 4 cc. of the buffered extract are quickly blown from a pipette into 4 cc. of the aqueous dye solution and the tube inserted into the machine within 5 seconds. A reading is made 15 seconds after the initial mixing (G_{s_1}) and again at 30 seconds (G_{s_2}). A small crystal of

² Owing to the differences in buffering power of tissues and the quantity used with a given volume of extractant, it is advisable to check roughly the final pH of the buffered extracts.

ascorbic acid is then added and a reading made after the dye has been completely reduced (*Gsr*). This gives a measure of the turbidity or color of the extract and is used to make a correction as follows: The machine is now adjusted so that a tube containing 4 cc. of 3 per cent metaphosphoric acid buffered to the same pH as the extract (Table II) plus 4 cc. of the dye and a crystal of ascorbic acid (completely reduced) reads the same as the reduced sample (*Gsr*). With the instrument set in this position, the concentration of the dye reagent (blank reading) is determined as follows: Mix 4 cc. of 3 per cent metaphosphoric acid buffered to the same pH as the extract with 4 cc. of the dye reagent and read in the instrument after 15 seconds (*Gb*).

The amount of ascorbic acid in the sample solution is calculated by use of the following equation in the manner previously described by Mindlin and Butler (9).

$$C = K_1 (\log G_s - \log G_b)$$

The value for K_1 was previously determined by treatment of buffered metaphosphoric acid solutions of pure ascorbic acid in the manner described above. These solutions were standardized by iodine titration (2). Under the conditions used K_1 was found to have a value of 0.088 ± 0.002 .

If there is an appreciable drift due to the slower reduction of the dye by substances other than ascorbic acid, the value G_s is obtained as follows:

$$G_s = G_{s_1} - (G_{s_2} - G_{s_1})$$

While this is necessarily an estimate, experience has shown (Table VI) this procedure to be quite accurate.

Dehydroascorbic acid is determined by bubbling H_2S slowly for 15 minutes through 5 to 7 cc. of the buffered extract in a 15 cc. conical centrifuge tube, allowing to stand 2 hours at room temperature, and freeing of H_2S by bubbling with wet tank nitrogen for 45 minutes to 1 hour. The total ascorbic acid is then determined as previously described. The difference in the two analyses represents the dehydroascorbic acid.

Results of Determinations by Method Presented

The close agreement (Table III) between determinations made on clear, colorless ascorbic acid solutions and on the same solu-

tions made turbid or colored by a variety of substances indicates the validity and precision of the method. The errors are of the same order as those found for duplicate tests on pure ascorbic acid solutions. The reduced sample and blank are conveniently read after 30 seconds reaction with an excess of ascorbic acid. At this time the reduced blank usually reads slightly less than the pure solvent. Preparation of a colorimeter tube containing extremely dilute India ink of such turbidity that it reads the same as the reduced blank can be used to avoid the necessity of preparing a reduced blank for each determination.

The procedure for plasma ascorbic acid previously described by Mindlin and Butler requires a crystal-clear filtrate. We suggest that the excess dye always be reduced and the blanks determined in the above manner. Plasma filtrates are not invariably clear even though they may appear to be. Tissue extracts are usually neither clear nor colorless. After many analyses on all sorts of tissues and fluids, we have found no exceptions to the performances demonstrated in Table III.

✓ The extract and reaction system is buffered at pH 3.5 to 3.7 for the following reasons: (a) this is a sufficiently low pH to insure reasonable specificity of the dye reaction, and (b) it is not acid enough to cause appreciable fading of the dye (9); (c) ascorbic acid and dehydroascorbic acid are both stable for several hours in this medium, and (d) the reduction of dehydroascorbic acid to ascorbic acid by H_2S under these conditions is rapid and practically complete.

Buffered metaphosphoric acid solution (pH 3.5 to 3.7) of ascorbic acid and dehydroascorbic acid, as well as extracts of several types of tissues containing added dehydroascorbic acid, shows no significant changes at 25° during periods up to 4 hours (Table IV).

✓ That the reduction of dehydroascorbic acid by H_2S is practically complete in less than 2 hours, at pH 3.5 to 3.7, is shown by experiments recorded in Table V. In more acid solutions than pH 3.0, the reaction becomes slow and incomplete. Decreasing stability and specificity preclude the use of the reaction at a pH higher than 4. The dehydroascorbic acid used was formed by iodine oxidation of a buffered metaphosphoric acid solution of ascorbic acid. The preparation of tissue extracts and treatment with H_2S was as described under "Procedure."

Under the conditions we have used, the treated solution is completely freed of H_2S by 30 to 40 minutes bubbling with wet tank nitrogen. Since this time varies with the nature of the sample, rate of bubbling, and many other factors, it should always be determined. Traces of H_2S not detectable by lead acetate paper cause large errors and rapid fading of the dye. This point is tested by periodically leading the exhaust nitrogen for 5 minutes

TABLE III

Determination of Ascorbic Acid in Turbid and Colored Solutions

Ascorbic acid originally present (a)	Substance added	Degree of turbidity or color; reading when reduced (Gr)	Ascorbic acid determined (b)	Error $100\left(\frac{b-a}{a}\right)$
mg. per 4 cc.			mg. per 4 cc.	
0.0232	No addition	99.0	0.0228	-1.7
0.0066	" "	99.0	0.0068	+3.0
0.0232	BaSO ₄	94.0	0.0227	-2.2
0.0076	"	91.3	0.0078	+2.6
0.0157	Glycogen	95.0	0.0155	-1.3
0.0220	"	90.3	0.0214	-2.7
0.0220	Tissue extract	92.0	0.0214	-2.7
0.0076	" "	89.5	0.0074	-2.6
0.0066	Green food dye (fast green FCF)	86.5	0.0067	+1.5
0.0220	" " " " " "	96.0	0.0226	+2.7
0.0218	Yellow food dye (tartrazine)	97.0	0.0215	-1.4
0.00636	" " " "	88.0	0.00636	0.0
0.0218	Red food dye (erythrosine)	78.0	0.0212	-2.8
0.00665	" " " "	65.0	0.00702	+5.6
0.0221	Beet pigment	50.0	0.0209	+5.4
0.0082	Radish pigment	86.0	0.0081	-1.2
0.0157	Carrot "	98.0	0.0155	-1.3

into a colorimeter tube containing dilute lead acetate solution. If black lead sulfide is not visible, test for turbidity in the colorimeter. When the galvanometer reads the same as for the reagents, we have never found trouble from residual H_2S .

One important feature of the photoelectric method for ascorbic acid determinations is the opportunity it offers to detect the presence of other reducing substances and therefore to estimate cor-

reactions for their effects. Mindlin and Butler recommended plotting the rate of drift on a log scale and extending the curve to

TABLE IV

Stability of Ascorbic Acid and Dehydroascorbic Acid in 3 Per Cent Metaphosphoric Acid Extracts Buffered to pH 3.5 to 3.7 with Citrate Buffer

Nature of extract	Ascorbic acid per 4 cc.		Dehydroascorbic acid per 4 cc.	
	0 hr.	4 hrs.	0 hr.	4 hrs.
	mg.	mg.	mg.	mg.
Ascorbic acid.....	0.0220	0.0217	0.00	0.00
Dehydroascorbic acid.....	0.0051	0.0054	0.210	0.206
New potato.....	0.0185	0.0181	0.0063	0.0060
Old ".....	0.0213	0.0220	0.0065	0.0062
Cauliflower.....	0.0172	0.0164	0.0086	0.0083
Spinach.....	0.0231	0.0228	0.00	0.00
Green beans.....	0.0134	0.0138	0.0072	0.0072
Rat liver.....	0.0162	0.0158	0.0081	0.0079
" kidney.....	0.0069	0.0066	0.0078	0.0074

TABLE V

Reduction of Dehydroascorbic Acid by H₂S in Buffered Metaphosphoric Acid, pH 3.5 to 3.7

Nature of solution	Dehydro-ascorbic acid originally present per 4 cc. (a)	Ascorbic acid after 2 hrs. per 4 cc. (b)	Ascorbic acid after 3 hrs. per 4 cc.	Per cent recovery $\frac{100 b}{a}$
	mg.	mg.	mg.	
Dehydroascorbic acid.....	0.0058	0.0056	0.0056	96.5
" ".....	0.0141	0.0138	0.0139	98.5
" ".....	0.0231	0.0224	0.0228	97.0
Old potato.....		0.0057	0.0055	
" ".....		0.0068	0.0067	
Potato + dehydroascorbic acid...	0.0202	0.0192	0.0197	95.0
Cauliflower.....		0.0074	0.0072	
" + dehydroascorbic acid.....	0.0209	0.0208	0.0206	99.5

zero time. We have followed the simple procedure of subtracting the drift recorded during the second 15 second period from the 15

second reading.³ In Table VI determinations made on pure ascorbic acid solutions are compared with solutions which show different rates of fading due to added —SH compounds. The agreement is good. It is necessary that the colorimeter tube be in place for at least 10 seconds before the first reading in order to avoid errors due to turbulence and bubbles.

TABLE VI

Ascorbic Acid Determinations in Presence of —SH Compounds at pH 3.5

Ascorbic acid originally present per 4 cc. (a)	Added substance	Rate of drift: divisions per 15 sec.	Ascorbic acid determined per 4 cc. (b)	Per cent error $100\left(\frac{b-a}{a}\right)$
mg.			mg.	
0	Glutathione 0.2 mm	0.7	0	
0.0064	" 0.2 "	0.7	0.0062	-3.1
0.185	" 0.2 "	0.7	0.0182	-1.6
0	Cysteine 0.2 mm	1.4	0	
0.0064	" 0.2 "	1.4	0.0066	+3.1
0.0185	" 0.2 "	1.4	0.0180	-2.7
0	<i>l</i> -Cysteinyl- <i>l</i> -cysteine 0.01 mm	6.0	0	*
0.0064	" " 0.01 "	6.0	0.0067	+4.7

¹ See (12).

DISCUSSION

Generally it has been assumed that the increase in titration value after treatment of tissue extracts with H₂S was due to the reduction of dehydroascorbic acid. The validity of the values reported in this paper is also dependent upon that assumption. Although direct proof is lacking, there are several facts in support of this postulate (Table VII). Those tissues which we have found to show extra reducing value after treatment with H₂S demonstrate this fact by reacting with the dye at a velocity as great as that of ascorbic acid. It is also significant that increased reducing power is usually found in those plant tissues which have been stored or harvested for a short time, which are still in good condition, but which are wilted or are not strictly fresh. Potatoes, when first mature, show practically no dehydro-

³ The reaction between ascorbic acid and the dye is complete in less than 15 seconds.

ascorbic acid, but after a few weeks storage, the dehydroascorbic acid gradually represents a significant part of the slowly decreasing total ascorbic acid. Such also is the case with cauliflower which has stood on the store shelf for a few days.

Dehydroascorbic acid seems to be destroyed nearly as rapidly as the ascorbic acid in the intact plant, since only a small part of the ascorbic acid decrease is represented by the dehydroascorbic

TABLE VII
*Ascorbic Acid and Dehydroascorbic Acid of Various Tissues by
Photoelectric Method*

Tissue	Ascorbic acid	Dehydro- ascorbic acid
	mg. per 100 gm.	mg. per 100 gm.
Fresh cauliflower.....	40	0
Storage cauliflower (10 days).....	29	4.7
Fresh spinach.....	45	0
Wilted spinach.....	10	1.5
Canned spinach.....	8.5	1
Fresh green beans.....	7.2	0
Wilted green beans.....	4.9	1.2
New potato (Katahdin).....	16.3	0.6
“ “ (Green Mountain).....	19.1	0
“ “ (Irish cobbler).....	14.3	0.5
Old “ (Green Mountain), 10 mos. storage.....	7.2	1.1
“ “ (Irish cobbler), 10 mos. storage.....	6.0	2.0
“ “ (Katahdin), 10 mos. storage.....	7.8	2.5
Strawberries.....	52	
Raspberries (red).....	25	
Blackberries.....	7.0	
Blueberries (North Carolina).....	6.0	
Beets.....	6.0	
Radishes (red).....	25	

acid value (Table VII). In wilted spinach which shows a large decrease in ascorbic acid, there appears only a small amount of the reversibly oxidized form. This fact no doubt accounts for the failure to detect differences between the antiscorbutic potency of foods and their titration values. The antiscorbutic effect of such small quantities of dehydroascorbic acid would lie within the experimental error of the biological method.

It has been adequately demonstrated (6, 7) that most animal

tissues contain a mechanism which reduces dehydroascorbic acid; therefore this form of the vitamin is probably not present in significant quantities in fresh tissue. However, analyses by the photoelectric method definitely show an increase in reducing value of liver and kidney extracts after H_2S treatment. This reaction velocity is comparable with that of ascorbic acid. The explanation for this observation seems to be that dehydroascorbic acid is formed in certain animal tissues during the process of extraction. The evidence that this increase in reducing value in animal tissue extracts is due to dehydroascorbic acid is not so strong as is the case with plant tissues, and needs further investigation. Whatever may be responsible for this extra reducing value in animal tissues, the photoelectric method offers a means of measurement.

Reedman and McHenry (8), as well as other investigators cited by these authors, have suggested the presence in certain plant tissues of an ascorbic acid compound, insoluble in the acid extractants ordinarily used but which liberates the soluble ascorbic acid on acid hydrolysis or heating. If this were true, it would be necessary to alter methods in which metaphosphoric acid is used so as to include the ascorbic acid from this hypothetical combination in the analysis.

We have been unable to confirm the existence of such an insoluble compound in either potatoes or cauliflower, supposedly two of the best sources. Also we have been unable to find a more complete or satisfactory extractant than 3 per cent metaphosphoric acid. Table VIII summarizes the result of the analyses of one variety of old potatoes and a sample of spinach after extraction by various methods. These results are typical of many determinations made on several varieties of both old and new potatoes, cauliflower, and spinach. The ascorbic acid values of potatoes or cauliflower which have been finely ground in HCl (0.2 to 1 per cent) under nitrogen do not change even after 10 hours hydrolysis. Heating the finely divided potato in nitrogen-saturated water 3 minutes before metaphosphoric acid extraction does not increase the total ascorbic acid but makes the sample easier to grind and usually leads to a slightly higher proportion of dehydroascorbic acid. Cold water extractions are practically as complete as with acids, although in this case most of the ascorbic acid appears in the reversibly oxidized form. Washed residues from acid extrac-

tions or precipitates obtained by acid treatment of aqueous extracts are free (chemically and biologically⁴) of ascorbic acid. However, if the sample is not finely ground and washed after extraction, there will remain a small amount of ascorbic acid in the residue. The use of small volumes of extracting medium (sometimes necessary in the titration technique) and failure to grind finely often lead to diffusion effects which might be misinterpreted as hydrolysis. We found no chemical or biological evidence to indicate that metaphosphoric acid extraction when properly carried out is incomplete.

TABLE VIII
Effects of Various Extraction Treatments on Ascorbic Acid Values

Tissue and method of extraction	Ascorbic acid	Dehydro-ascorbic acid	Total
	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.
Potato, 3% metaphosphoric acid	7.2	1.4	8.6
“ 1% HCl, cold	5.2	3.4	8.6
“ 1% “ “ after 2 hrs. (hydrolysis).....	5.2	3.4	8.6
Potato 1% HCl, hot.....	7.2	1.3	8.5
“ water, hot.....	7.0	1.3	8.3
“ “ cold.....	1.3	7.1	8.4
Spinach, 3% metaphosphoric acid	46.4	0	46.4
“ water, cold.....	0.0	45.1	45.1
“ “ hot.....	39.8	6.8	46.6
“ 1% HCl.....	44.1	2.0	46.1

All determinations reported in Table VIII were made on solutions buffered to pH 3.5 just previous to analysis. Ascorbic acid determinations made at widely different pH values, especially in tissue extract, are not comparable. Solutions made from reagent quality HCl or H₂SO₄ react with the dye even when properly buffered and therefore are not recommended for general use. The blank determination must be made at the same pH (within 0.1) and in the same acid medium as the extract. HCl solutions buffered to the same pH as metaphosphoric acid do not show the

⁴ Tested in the usual manner by growth response and pathological healing in the guinea pig.

same color intensity. If cyanide is used, it also must be added to the blank or errors will result, owing to independent reaction with the dye.

In Table VII are reported the ascorbic acid analyses of several highly colored tissue extracts which have been previously impossible by chemical methods. These values are in good agreement with previously reported biological tests (3).

Ascorbic Acid in Urine

Satisfactory ascorbic acid determinations in urine have been difficult because of the yellow color and rapid fading of the dye, especially in specimens of low ascorbic acid value. These troubles are largely eliminated in the photoelectric method.

An equal volume of 6 per cent metaphosphoric acid is added to 25 cc. of urine and centrifuged or filtered if not clear.⁵ To 25 cc. of the acid urine are added 7 cc. of citrate buffer. Run the buffered urine from a 5 cc. graduated pipette into 4 cc. of the dye solution until the dye is completely reduced. Then estimate roughly the required dilution so that 4 cc. of the buffered urine will incompletely reduce 4 cc. of the dye. Dilute an aliquot of the buffered urine with metaphosphoric acid-citrate mixture (25:7) to the right concentration and determine the ascorbic acid by the procedure previously described. Such a variety of reducing substances other than ascorbic acid occurs in the urine that it is difficult to eliminate the effects of all of them. Those substances which reduce the dye at a slower rate than ascorbic acid have been eliminated by the above method. However, thiosulfate, which reacts rapidly with the dye, may be included in the analysis. Like previous urine methods this procedure is most useful in comparing the excretion value before and after the administration of ascorbic acid.

SUMMARY

1. A photoelectric indophenol method has been described for the determination of small quantities of ascorbic acid and dehydro-ascorbic acid in turbid or colored extracts.

⁵ This ascorbic acid in solution will remain stable for several hours in the ice box.

2. Proof for the validity and precision of each step of the method is presented.

3. Determinations show a very good precision for small quantities of ascorbic acid (6 to 20 micrograms).

4. Estimation of the extent of interference from other reducing substances is possible.

5. The method is applicable to a wide variety of plant and animal tissues and fluids.

6. Determinations of ascorbic acid and dehydroascorbic acid after various methods of extraction are presented and no evidence is found that metaphosphoric acid extractions are incomplete.

7. Ascorbic acid and dehydroascorbic acid analyses for several vegetables are given. Determinations in beets and highly colored berry juices are included.

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THE COMPOSITION OF SPHINGOMYELIN FROM STROMATA OF RED BLOOD CELLS*

By S. J. THANNHAUSER, PAUL SETZ, AND JOSEPH BENOTTI

(From the Department of Medicine, New England Medical Center and Tufts College Medical School, Boston)

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Bürger and Beumer (1) found that the stromata of red blood cells contained diaminophosphatide (sphingomyelin). These authors estimated the amount present in stromata to be approximately 6 per cent of the dry weight. Thannhauser and Setz (2) found, quantitatively, between 4 and 5 per cent. The composition of sphingomyelin of the red blood cells was previously unknown. The purpose of this work was to isolate sphingomyelin in pure form from stromata and to establish its composition.

Procedure

Defibrinated bovine blood in batches of 40 to 50 liters was centrifuged in a large Sharples centrifuge. 5 liters of centrifuged red blood cells were suspended in 40 liters of water to which had been added 35 gm. of sodium chloride. The mixture was centrifuged a second time and the centrifugate hemolized in tap water. After adding 25 gm. of sodium chloride, the suspension of red blood cells was centrifuged a third time. 6 to 7 liters of acetone were added to the centrifugate which was then placed on ice for 3 days. At the end of this period the supernatant liquid was siphoned off and the residue dried in a vacuum drying oven. The dry material was then ground in a stone mill.

The dry stromata were extracted in a large extraction apparatus, described by Fränkel and Pollanz (3), with petroleum ether for 48 hours. This was followed by an ether extraction for a similar period. Concentration of the petroleum ether extract produced a

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substance which gave negative nitrogen, phosphorus, and Molisch reactions. By analysis the substance was shown to be largely cholesterol. There was a trace of nitrogen and phosphorus found in the ether extract. A weak Molisch reaction was also obtained. Further investigation of this extract was thought unwarranted.

The stromata were then extracted with cold 95 per cent alcohol. The alcoholic extract was fractionated into three parts by concentration in a vacuum. Each fraction contained N, P, and cholesterol. At that time the separation of sphingomyelin by means of Reinecke acid (4) was unknown. Thus it was impossible to separate the diaminophosphatides from the monoaminophosphatides. All efforts to obtain pure sphingomyelin from this cold extract were unsuccessful.

The material was next extracted with 95 per cent alcohol at 55° for several hours, at least three times. The pooled extracts were concentrated to a small volume and placed in the ice box for several days. A brownish colored mass settled to the bottom. This precipitate was treated with ether, followed by acetone to remove some of its color, and then recrystallized from alcohol. This yielded a yellowish solid substance which contained 5.3 per cent cholesterol, 2.7 per cent N, and 3.5 per cent P (N:P, 2:1.16). The Molisch reaction was slightly positive. The substance was treated with hot acetone to remove cholesterol, thereby reducing the cholesterol content to 3.3 per cent. Finally the substance was mixed with sand and extracted with ether for 48 hours in a Soxhlet extractor. The contents of the thimble were dissolved in methanol-petroleum ether (1:10), thus separating it from the sand. It was then passed through a column of aluminum oxide. In this manner the substance was decolorized and rendered free of cerebrosides, since the latter are retained in small quantities by aluminum oxide (4). The colorless solution yielded, after evaporation in a vacuum, a white substance, which was free of cholesterol, and cerebrosides containing 3.6 per cent N, 3.94 per cent P (N:P, 2:1.1). The latter substance was dissolved in warm methanol, placed in the ice box overnight, and then filtered. This substance gave the following analysis:

2.845 mg. substance:	6.97 mg. CO ₂ ,	2.97 mg. H ₂ O
5.412 " "	: 0.157 cc. N ₂ ,	738 mm., 19°
6.980 " "	: 17.280 mg. phosphomolybdate	

Palmitic sphingomyelin,	$C_{55}H_{101}N_2PO_7$.	Calculated.	C 64.96, H 11.19, N 3.86, P 4.30
Stearic	" $C_{61}H_{109}N_2PO_7$.	Calculated.	C 65.71, H 11.44, N 3.74, P 4.15
Lignoceric	" $C_{67}H_{117}N_2PO_7$.	Calculated.	C 67.72, H 11.72, N 3.36, P 3.73
		Found.	C 66.82, H 11.68, N 3.29, P 3.60

When acetone was added to the mother liquor, a snow-white precipitate resulted. The acetone-precipitated substance had the same crystalline structure (long bent needles) as the fraction obtained at 0°.

Hydrolysis of Sphingomyelin from Stromata of Red Blood Cells

The hydrolysis of the sphingomyelin, the isolation of the methyl-esters of the fatty acids, and their distillation were carried out as previously described by Fränkel, Bielschowsky, and Thannhauser (5). The molecular weights of the fatty acids were determined by titrating against alcoholic KOH. The iodine numbers were determined by the method of Rosenmund and Kuhnhehn (6). The *p*-phenyl phenacyl esters were prepared as described by Thannhauser and Benotti (7).

Unlike the sphingomyelins obtained from other organs, a large precipitate settled out from the hydrolysis mixture kept overnight. This was called Part A. The mother liquor from the above precipitate was called Part B. (See Table I.)

The theoretical amount of methyl ester calculated for lignoceric acid would be 900 mg. Our yield was 449 mg. from Part A and 403 mg. from Part B, giving a total of 852 mg. of methyl esters.

Part A proved to be lignoceric methyl ester. Part B was divided into two fractions. One fraction consisted of a small amount of fatty acid with a low melting point. This appeared, according to the melting point of both the free acid and *p*-phenyl phenacyl ester to be a mixture of palmitic and stearic acids. However, the iodine numbers 24 and 29 for Fractions 1 and 2 indicate that there are some small quantities of unsaturated substances present which may be traces of unsaturated fatty acids. The larger fraction contained an acid with a higher melting point. The melting point of its *p*-phenyl phenacyl ester, 104° (pure, 105.5°), indicates that the latter substance was largely lignoceric acid.

TABLE I

Distillation of Crude Methyl Esters from 1.989 Gm. of Sphingomyelin

Fraction No.	Outside temperature	Inside temperature	Pressure	Weight of free acid	M.p. of free acid	Mol. wt.	I No.	p-Phenyl phenacyl ester
Part A, 449 mg. methyl ester								
1	°C. 195-200	°C. 135-140	mm. 0.01-0.015	mg. 370	°C. 83.5*	378		°C. 105.5
Part B, 403 mg. methyl ester								
1	144-176	90- 98	0.015	88	65	275	24	91-92†
2	188-210	95-100	0.01	198	78	324	29	104

* Repeated recrystallization from dioxane. Melting point of synthetic lignoceric acid, 84.5° (8). Chibnall, Piper, and Williams (8) examined lignoceric acid obtained by splitting kersasin from brain. Measuring the long crystal spacing in A. by x-ray photograph, they concluded that their preparation of lignoceric acid from kersasin is contaminated with 10 per cent C₂₂, 10 per cent C₂₆ fatty acids. The lignoceric acid from natural products melts at 81°. They would like to maintain the name lignoceric acid only for the synthetic acid (m.p. 84.5°) and to replace the name "lignoceric" from the naturally occurring substance (m.p. 81°) to "mixed fatty acids."

† Not enough for recrystallization.

DISCUSSION

It is known that the lipid composition of organs differs from that found in red blood cells. In contrast, red blood cells contain cholesterol only in the free form and not as cholesterol esters (Wacker and Hueck (9)).

The proportion of diaminophosphatide to monoaminophosphatide in other organs, for example spleen and liver, is roughly 1:5 (unpublished data). In the stromata we found a proportion of 1:2.

This work demonstrates that the composition of the sphingomyelin of stromata varies from that of other organs. Thannhauser and Benotti (7) have shown that spleen contains palmitic, stearic, and lignoceric sphingomyelin in the ratio of 2:2:1 (average). The analysis of sphingomyelin from red blood cells shows that it is composed largely of lignoceric sphingomyelin. This fact may have a physiological significance which we are unable to explain at present.

SUMMARY

A method has been described for the preparation of sphingomyelin from large quantities of stromata from red blood cells. The sphingomyelin obtained was hydrolyzed. The larger fraction proved to be lignoceric sphingomyelin.

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